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**Quinoprotein dehydrogenase and pyrroloquinoline quinone modified
enzyme electrodes**

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" ...ach cum creideamh nad bheatha, sonas nad chridhe

Tha an lasair nad anam aig meadhan do bhith "

ABSTRACT

This study is a qualitative investigation into the experiences of young people who have been involved in the criminal justice system. The research was conducted using semi-structured interviews with 12 participants, aged 16-25, who had been in contact with the police or the courts in the last 12 months. The findings suggest that young people often feel stigmatised and marginalised after their involvement with the criminal justice system, and that they may experience difficulties in finding employment and housing. The study also highlights the importance of providing support and guidance to young people who have been involved in the criminal justice system, in order to help them to reintegrate into society and avoid further involvement with the criminal justice system.

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For my family: Dad, Mum, John, Angela, Liam and Kevin

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ABSTRACT

This thesis concerns the use of the coenzyme PQQ and quinoprotein dehydrogenase enzymes for the development of enzyme electrodes. A general introduction to the area which describes the properties of quinoprotein dehydrogenases is given. The reactivity and redox properties of the quinone cofactor, PQQ, are also outlined.

The ammonium ion requirement and stability of the quinoprotein methanol dehydrogenase were investigated. A packed cavity electrode was used with the organic conducting salt TTF.TCNQ. Methanol dehydrogenase isolated from *Paracoccus denitrificans* was more stable than enzyme isolated from *Methylophilus methylotrophus* and was successfully used for repeated assay in packed cavity electrodes without significant loss in current output. These investigations also showed that, contrary to suggestions in the literature, ammonium ions are necessary for efficient re-oxidation of methanol dehydrogenase at the organic conducting salt electrode.

Methylamine dehydrogenase was used in conjunction with the insoluble mediator tetracyanoquinodimethane (TCNQ) to construct enzyme electrodes which will potentially provide a simple, rapid method for analysis of histamine without the need for extensive sample pretreatments currently required in HPLC and GLC analysis. The linear response of this amperometric sensor, between 0 and 150 μM , correlates well with elevated histamine levels predominant in patients with chronic myelogenous leukaemia. The observed limit of detection, 4.8 μM , compares favourably with the lower limits of detection reported for a potentiometric histamine sensitive enzyme electrode.

The electrochemistry of PQQ was examined and the coupling of PQQ with glucose oxidase was studied at different pH values. Optimal interaction between PQQ and glucose oxidase was observed at pH 3.5. Results from these studies enabled construction of PQQ-glucose oxidase modified enzyme electrodes for the detection of glucose in RibenaTM blackcurrant juice. The corresponding amperometric determination of glucose was in good agreement with results obtained using commercial enzymatic test kits supplied by Boehringer Mannheim and Sigma Diagnostics.

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LIST OF ABBREVIATIONS

ADH	Quinoprotein Alcohol Dehydrogenase
CE	Counter Electrode
CME	Chemically Modified Electrode
DCPIP	2,6-Dichloro Phenol Indophenol
EDTA	Ethylene Diamine Tetra Acetic Acid
FAD	Flavin Adenine Dinucleotide
FDH	Quinoprotein Fructose Dehydrogenase
GDH	Quinoprotein Glucose Dehydrogenase
GOD	Glucose Oxidase
MADH	Quinoprotein Methylamine Dehydrogenase
MDH	Quinoprotein Methanol Dehydrogenase
NAD(P) ⁺	Nicotinamide Adenine Dinucleotide (Phosphate)
OCS	Organic Conducting Salt
PES	Phenazine Ethosulphate
PMS	Phenazine Methosulphate
PPy	Poly(Pyrrole)
PQQ	Pyrrolo Quinoline Quinone
PQQH [•]	Pyrrolo Quinoline Semi Quinone
PQQH ₂	Pyrrolo Quinoline Quinol
PQQTME	Pyrrolo Quinoline Quinone Tri Methyl Ester
PS	Poly(Styrene)
PVC	Poly(Vinyl Chloride)
PTFE	Poly(Tetra Fluoro Ethylene)

RE	Reference Electrode
SCE	Saturated Calomel Electrode
TCNQ	Tetracyanoquinodimethane
TMPD	N,N,N',N'-Tetra Methyl-4-Phenylene Diamine
Tris	Tris(Hydroxymethyl)Aminomethane
TTF	Tetrathiafulvalene
TTF.TCNQ	Tetrathiafulvalene-Tetracyanoquinodimethane Conducting Salt
TTQ	Tryptophan Tryptophan Quinone
WE	Working Electrode

LIST OF SYMBOLS

A	electrode area (cm^2)
D	diffusion coefficient (cm.s^{-1})
E	potential vs a reference electrode (V)
$E_{1/2}$	half-wave potential
ΔE_p	peak to peak separation
F	Faraday constant ($9.6485 \times 10^4 \text{ C.mol}^{-1}$)
i	current (A)
I	current density (A.cm^{-2})
K_M	Michaelis constant (mol.dm^{-3})
Q	charge (C)
t	time (s)
v	potential scan rate (V.s^{-1})

1. QUINOPROTEIN DEHYDROGENASES AND BIOSENSOR DEVELOPMENT

1.1 GENERAL INTRODUCTION

This thesis concerns the use of NAD(P)⁺-independent quinoprotein dehydrogenases as an alternative group of enzymes for biosensor development. There are many properties of quinoprotein dehydrogenases which could make them more amenable for incorporation into biosensors than enzymes such as glucose oxidase and NAD(P)⁺-dependent dehydrogenases which are usually used. These include :

- i) the absence of oxygen requirement for enzymatic reaction removes problems of oxygen interference which is the principal disadvantage of glucose oxidase biosensors (Cass *et al.*, 1984);
- ii) no requirement for an external NAD(P)⁺ cofactor which considerably complicates the construction of practical devices based on the pyridine nucleotide-dependent dehydrogenase enzymes (Turner, 1988);
- iii) a redox dye-linked quinone prosthetic group enables the construction of both electrochemical and optical quinoprotein dehydrogenase enzyme electrodes;
- iv) the very high catalytic activity, as illustrated by glucose dehydrogenase (GDH turnover number 320 000 min⁻¹) could enable the development of small sensors with a high current density.

However, the amenable properties of quinoprotein dehydrogenases must be offset by the relative instability of quinone-dependent glucose and methanol dehydrogenase and the

restricted availability of quinoprotein dehydrogenases.

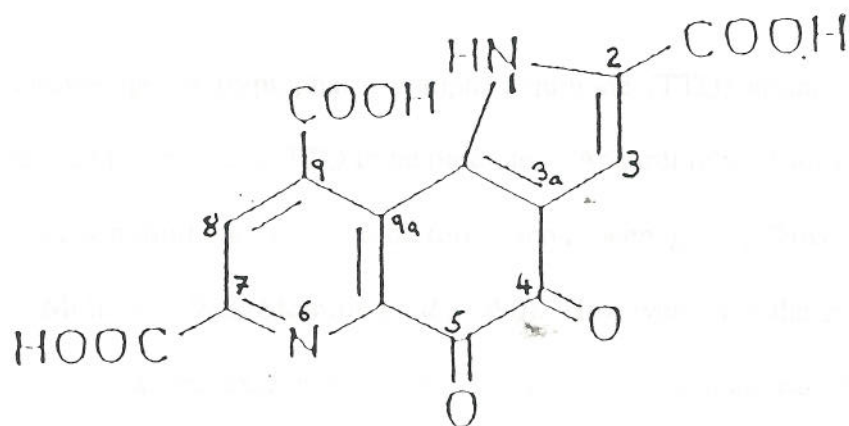
1.2 THE QUINONE PROSTHETIC GROUP

Pyrroloquinoline quinone (PQQ) is the semi-systematic name of the stable coenzyme found in most quinoprotein dehydrogenases (Duine *et al.*, 1980). Electron spin resonance spectra revealed the quinone nature of this prosthetic group (Salisbury *et al.*, 1979; Westerling *et al.*, 1979), and its chemical structure: 2,7,9 -tricarboxy-1H-pyrrolo (2,3 f) quinoline-4,5-dione was resolved by Duine *et al.* in 1980 (see Figure 1.1.1). Initially purified from *Hyphomicrobium* X (Duine & Frank, 1979; Duine *et al.*, 1986), PQQ has been identified in a wide range of micro-organisms (Duine *et al.*, 1986). Characterised quinoprotein dehydrogenases include: methanol dehydrogenase (MDH) (Duine *et al.*, 1978; Duine & Frank, 1979), glucose dehydrogenase (GDH) (Duine *et al.*, 1979b; Duine *et al.*, 1982) and fructose dehydrogenase (FDH) (Yamada *et al.*, 1966; 1967; Ameyama *et al.*, 1981b).

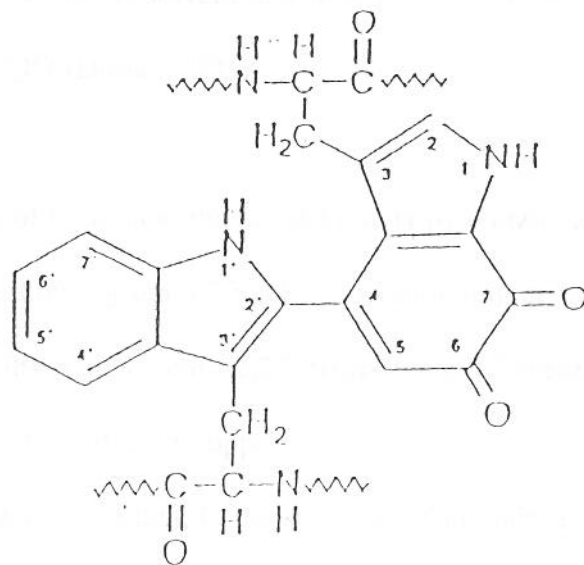
The mode of binding of the PQQ prosthetic group to quinoproteins was studied by Ameyama *et al.* in 1985. These studies indicated the essential role of magnesium ions in anchoring PQQ to the apo-glucose dehydrogenase. PQQ dissociates easily when dialysed against EDTA containing buffers. Holoenzymes (with PQQ) can then be prepared by dialysing apoenzymes (without PQQ) against PQQ and Mg^{2+} -containing buffers. Reversible thermal inactivation of holoenzyme GDH has also been demonstrated at temperatures above 35°C. Calcium ions were essential for the re-activation of active holoenzyme at 25°C (Geiger & Gorisch, 1989). The presence of

two Ca^{2+} ions per subunit of GDH indicates that PQQ is bound at the active site of the enzyme via a Ca^{2+} bridge which stabilizes GDH against thermal inactivation. Previously characterised reactions of methanol dehydrogenase-bound PQQ with oxygen (Davidson *et al.*, 1992a) provided evidence for a structural role for calcium (Davidson *et al.*, 1992b) in this enzyme. The functional importance of Ca^{2+} in methanol dehydrogenase was emphasized by the fact that at least three gene products of the *mox* gene cluster are required for its incorporation into the enzyme (Ghosh & Anthony, 1992). Methanol dehydrogenase samples isolated from mutants defective in these genes lack Ca^{2+} and contain a full complement of PQQ, but they are inactive and exhibit perturbed PQQ absorption spectra. The positive charge on the Ca^{2+} in the wild-type enzyme may have an inductive effect on the redox potential of PQQ, helping to stabilize the reduced and semiquinone forms of the enzyme (Davidson *et al.*, 1992b).

The amine oxidoreductase methylamine dehydrogenase (MADH), first isolated by Eady and Large in 1968, was previously thought to contain covalently bound PQQ (de Beer *et al.* 1980; Husain & Davidson, 1987). However, the three dimensional structure of MADH, resolved at 2.5 Å, indicated that the active site could not be fitted with PQQ (Vellieux *et al.*, 1989a). On this evidence Vellieux *et al.* suggested that the cofactor of MADH might be a precursor of PQQ, and subsequently proposed a new model for the pro-PQQ cofactor of *Thiobacillus versutus* methylamine dehydrogenase (Vellieux *et al.*, 1989b). Structural investigations of methylamine dehydrogenase, isolated from several different bacteria, have now revealed that the cofactor of MADH is : 4-(2'-tryptophyl)-tryptophan-6,7-dione (TTQ) (McIntire *et al.*, 1991; Chen *et al.*, 1991). This



1.1.1



1.1.2

Figure 1.1.1 The chemical structure of pyrroloquinoline quinone (PQQ)

Figure 1.1.2 The chemical structure of tryptophan tryptophan quinone (TTQ)

quinoid aromatic amino acid (Figure 1.1.2), consists of a tryptophan dimer with an *o*-quinone group (Duine 1991).

Current knowledge of tryptophan-tryptophan quinone (TTQ) amine oxidoreductases enables several properties of TTQ to be predicted. Accordingly, absorption maxima for the quinone, semiquinone and quinol forms have been given, (Husain *et al.*, 1987; Kenney & McIntire, 1983; McIntire *et al.*, 1990). However, from the three dimensional structure of these amine oxidoreductases (Chen *et al.*, 1991), it appears that the covalent bridge between the two tryptophans has a dihedral angle of 45° (Kenney & McIntire, 1983). Consequently the tryptophyl rings are not coplanar, preventing resonance enhancement, therefore the absorbance in the visible region is mainly derived from the *o*-quinone moiety. Based on this structural data, the carbonyl group at the C₆ position of TTQ is most accessible to solvent and may therefore have a similar role to the C₅ carbonyl group in PQQ (Duine, 1991).

Subsequent analysis of hydrazine-modified MADH by Huizinga *et al.* (1992) identified C₆ as the reactive carbonyl group of TTQ. Corresponding crystal structures of MADH inhibited by methylhydrazine and (2,2,2-trifluoroethyl)hydrazine revealed enhanced electron density attached to C₆ as opposed to the C₇ atom of TTQ. This result is in good agreement with the fact that, in the structure of uninhibited MADH, C₆ but not C₇ is exposed to the active site of MADH (Huizinga *et al.*, 1992).

1.3 CHARACTERISED QUINOPROTEIN DEHYDROGENASES

1.3.1 Methanol dehydrogenase

Most methanol dehydrogenases are composed of two subunits and have a molecular weight of 120 000 (Duine *et al.*, 1987). Methanol dehydrogenase (EC. 1.1.99.8) oxidises a wide range of primary alcohols via the natural electron acceptor cytochrome c_L (Duine & Jongejan, 1989):



Substrate specificity depends on the source of MDH, some enzymes oxidise both primary and secondary aliphatic alcohols (Bamforth & Quayle, 1978), others aromatic alcohols (Yamanaka & Tsuyuki, 1983), but oxidation of aldehydes is restricted to formaldehyde. An alkaline pH and ammonium salts are required for the *in vitro* enzyme assay where cytochrome c_L can be replaced by several artificial electron acceptors including: phenazine ethosulphate (PES), phenazine methosulphate (PMS) and Wurster's blue (Duine & Jongejan 1989). With Wurster's blue as the electron acceptor (50 mM NH_4Cl as activator) an apparent K_M of 0.3 mM is found for methanol (Frank & Duine 1990).

1.3.2 Glucose dehydrogenase

Quinoprotein glucose dehydrogenase (GDH) (EC.1.1.99.17) oxidizes monosaccharides and disaccharides to the corresponding lactones.



Both soluble and membrane-bound GDH have been isolated from *Acinetobacter calcoaceticus*, and shown to be different in all aspects, i.e., optimum pH, kinetics, substrate specificity and molecular size (Matsushita *et al.*, 1989b). Soluble GDH is a dimer composed of two identical subunits of M_r 48-54 000 and each subunit contains one molecule of PQQ (Dokter *et al.*, 1986; Geiger and Gorisch, 1986). In contrast, the membrane-bound GDH is a single polypeptide of M_r 83-87 000 (Matsushita *et al.*, 1988; 1989b).

1.3.3 Fructose dehydrogenase

Fructose dehydrogenase (FDH) is another important PQQ enzyme, but the structure and location of the prosthetic group in the holoenzyme are still unknown. Isolated from *Gluconobacter* species, fructose dehydrogenase (EC.1.1.99.17) has a molecular weight of 140,000. The optimum pH for fructose oxidation is 4.0 and an apparent K_M of 10^{-2}M fructose has been determined for purified FDH (Ameyama *et al.*, 1981b; 1992). Upon enzymatic oxidation of D-fructose, the prosthetic group (PQQ) is reduced to PQQH₂ and then an electron acceptor re-oxidizes PQQH₂ to PQQ, liberating two electrons. This oxidation of D-fructose may occur *in vitro* using redox dyes such as ferricyanide,

2,6-dichlorophenol indophenol (DCPIP) or PMS (Ameyama *et al.*, 1981b). Alternatively, Khan *et al.* (1991) illustrated direct electron transfer of FDH at metallic electrodes in accordance with the following equations:



1.3.4 Methylamine dehydrogenase

Methylamine dehydrogenase (MADH) is an oligomeric protein composed of large and small subunits (molecular weights 46 700 and 15 500 respectively) joined in an $\alpha 2\beta 2$ configuration (de Beer *et al.*, 1980). The quinoid cofactor (TTQ) is composed of two tryptophyl residues from the L-subunit of MADH covalently linked through a C₂-C₄ bond (Chistoserdov *et al.*, 1990). At present nothing is known about the biochemistry of the cofactor formation.

Methylamine dehydrogenase (EC.1.4.99.3) has a very broad substrate specificity, oxidizing primary amines to the corresponding aldehydes and ammonia (Duine *et al.*, 1987):



The reaction proceeds with methylamine and either PMS, or PES artificial electron acceptors, via a ping-pong mechanism in which the aldehyde product is released from the enzyme prior to the interaction with reoxidant (Davidson, 1989). Optimal enzyme activity occurs at pH 7.5, with K_M values of 10 μM for methylamine and 300 μM for PES, and a V_{max} of 16.9 $\mu\text{mol}\cdot\text{min}^{-1}$ per mg of protein (Husain & Davidson, 1987; Davidson, 1989).

1.4 REACTIVITY OF PYRROLOQUINOLINE QUINONE

The quinone carbonyl group of PQQ is easily attacked by nucleophiles such as amines, amino acids, hydrazines, and thiols to form a covalent adduct. The presence of a pyridine nucleus in PQQ facilitates the nucleophilic addition of amines to the C_5 quinone carbon atom and stabilizes the carbinolamine intermediate formed by intramolecular hydrogen bonding. The presence of an acidic pyrrole proton is also very important for intramolecular general base catalysis to occur (Itoh, 1992).

Upon addition of water, acetone, and acetaldehyde, PQQ is transformed into a strongly fluorescing compound which is hydrated at the C_5 position (PQQ- H_2O). Further hydration of PQQ- H_2O occurs at the C_4 position producing dihydrate (PQQ- $2\text{H}_2\text{O}$) (Dekker *et al.*, 1982). PQQ also reacts with quinoprotein enzyme substrates and activators. Corresponding spectra revealed the formation of 5-alkoxy-5-hydroxy PQQ and 5-amino-5-hydroxy PQQ when incubated with alcohols and 2M NH_4Cl respectively, (Dekker *et al.*, 1982).

Alcohols add to the quinone moiety of PQQ reversibly to form a hemiketal-type adduct, but no redox reaction is observed *in vitro* (Itoh *et al.*, 1985b). The addition position of water and alcohols has always been considered to be C₅, but without any direct evidence. However, Itoh *et al.* (1991a) showed that methanol addition occurs at C₄ depending on reaction conditions. The pyridine nucleus of PQQ and the ester groups on it considerably enhance adduct formation. In general, hydration or alcohol addition to carbonyl compounds is largely enhanced when the carbonyl compounds have a highly electron-withdrawing substituent. The pyrrole ring, on the other hand, showed an opposite effect, which can be attributed to its electron-releasing nature.

An Oppenauer-type oxidation of alcohols by PQQ trimethylester (PQQTME) using aluminium salts as a mediator has been reported by Itoh *et al.* (1985b; 1987). An aluminium alkoxide formed from an alcohol and aluminium chloride *in situ* interacts with the more electron rich quinone carbonyl oxygen (C₄) of PQQTME. Co-ordination of the aluminium counter cation to the quinone carbonyl as a Lewis acid continues the oxidation reaction via removal of a net hydride ion from the quinone (Itoh *et al.*, 1985b; 1987).

The oxidation of primary amines such as cyclohexamine and glycine by quinones produces 6-amino-5-hydroxyphenanthroline, or aminophenol, as the sole isolatable reduced product. In contrast the reactivities of secondary and tertiary amines are relatively low. These findings, together with observations of general base catalysis by the amine substrate itself and of consecutive first-order kinetics, suggest that the

oxidation of primary amines proceeds via a transamination mechanism that involves a C₅ carbinolamine intermediate (Itoh *et al.*, 1991a). Once formed, the carbinolamine intermediate is converted via two competing reaction pathways, i.e., α -deprotonation and transamination, to give a quinol and an amino phenol, respectively.

At a low amine concentration, α -deprotonation of the carbinolamine intermediate (addition-elimination) predominates to give the quinol. On the other hand, when a relatively large amount of amine is present, the iminoquinone intermediate, derived via a similar addition-elimination sequence from the propyl-amino-phenol product accumulates. This ionic mechanism has been further confirmed by examining the reaction using PQQTME in organic media, where the iminoquinone-type intermediate and the quinol and aminophenol products are isolated and well characterized (Itoh *et al.*, 1991a).

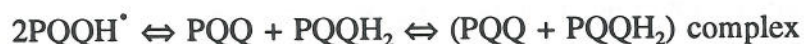
The carbinolamine-type adduct is also considered to be a key intermediate from which the redox reaction (quinol formation) proceeds via electron flow from the hydrazino nitrogen to the quinone. The reduction of PQQ proceeds via formation of the carbinolamine-type intermediate which is followed by electron flow from the nitrogen of hydrazines into the quinone moiety of PQQ. If such electron flow is not fast enough, dehydration from the intermediate predominantly proceeds to give the C₅ hydrazone or azo adduct (Itoh, 1992).

Electron-withdrawing substituents would retard such electron flow as in 4-

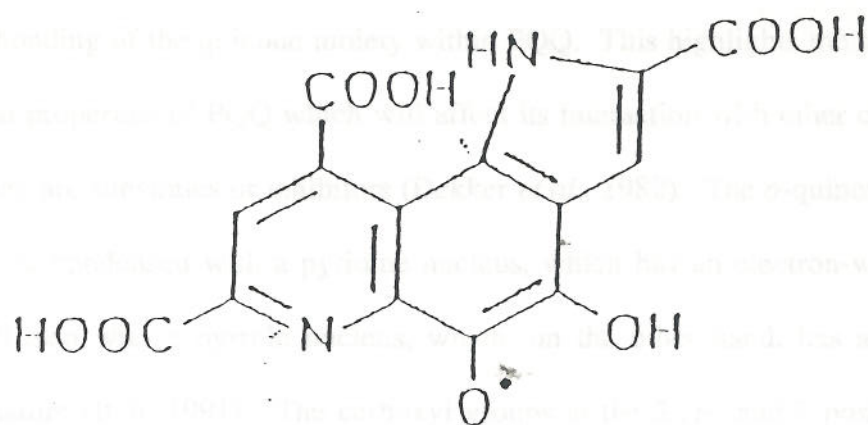
nitrophenylhydrazine and 2,4-dinitrophenylhydrazine to give the corresponding adducts where as electron-donating substituents such as methyl and phenyl facilitate the reduction of PQQ. The importance of the electron-withdrawing effect of the substituent attached to the hydrazino group is also found in the reaction between PQQ and semicarbazide and acetohydrazide; the azo adducts are the major products even at higher pH conditions. In conclusion, the more electron-withdrawing nature of the substituent, the more preferable is the adduct formation (Itoh & Ohshiro, 1993).

1.5 REDOX PROPERTIES OF PYRROLOQUINOLINE QUINONE

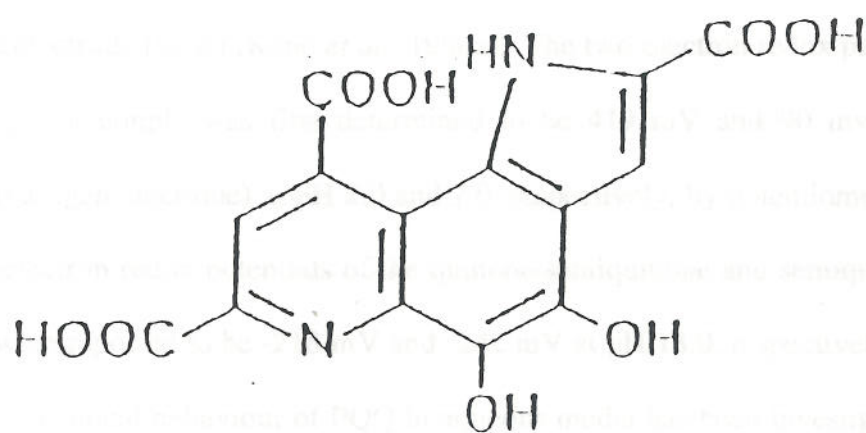
Biologically relevant redox forms of the PQQ prosthetic group include the semiquinone radical ($\text{PQQH}^\bullet$) and reduced quinol (PQQH_2) which are illustrated in Figures 1.2.1 and Figure 1.2.2. Further reduction with NaBH_4 in the presence of oxygen produces the tetrahydrate PQQH_4 . Eckert and Bruice (1982) first characterised the electrochemical /chemical properties of "methoxatin" now identified as PQQ. They observed that PQQ reduction corresponded to a minimum of 3 redox potentials each of which is not equivalent to the transfer of a single electron. The equilibrium of this incomplete electron transfer was thought to involve reversible interaction of $2\text{PQQH}^\bullet$ with PQQ and PQQH_2 via an intermediate PQQ-PQQH_2 complex (Eckert & Bruice, 1982).



The pyridine N of the quinone group influences the stability of carbonyl adducts and



1.2.1



1.2.2

Figure 1.2 Redox forms of the PQQ prosthetic group

Figure 1.2.1 PQQH• Pyrrolo-quinoline semiquinone

Figure 1.2.2 PQQH₂ Pyrrolo-quinoline quinol

the rate of carbonyl reduction via inductive withdrawal of electrons and internal hydrogen bonding of the quinone moiety within PQQ. This highlights the significance of chemical properties of PQQ which will affect its interaction with other compounds, whether they are substrates or inhibitors (Dekker *et al.*, 1982). The *o*-quinone ring, the active site, is condensed with a pyridine nucleus, which has an electron-withdrawing nature, and also with a pyrrole nucleus, which, on the other hand, has an electron-donating nature (Itoh, 1991). The carboxyl groups at the 2-, 7-, and 9-positions must play very important roles not only for binding to the active site of quinoproteins but also for the reactivity of PQQ itself.

The redox couple corresponding to the oxidation and reduction of PQQ at a mercury electrode, at pH 7.0, was determined at -175 mV with respect to a saturated calomel reference electrode (SCE) (Kano *et al.*, 1990a). The two-electron redox potential of the quinone/quinol couple was first determined to be 419 mV and 90 mv (vs N.H.E., normal hydrogen electrode) at pH 2.0 and 7.0, respectively, by potentiometric titration. The one-electron redox potentials of the quinone/semiquinone and semiquinone quinol couples were reported to be -218 mV and -242 mV at pH 13.0, respectively. Recently, the electrochemical behaviour of PQQ in aqueous media has been investigated in more detail by cyclic voltammetry. The single electron redox potentials for the quinone/semiquinone and semiquinone/quinol couples in a wider pH range (6-12) were calculated by computer simulation. The PQQ-semiquinone is generated by electrochemical reduction under alkaline conditions (pH 12) and is well characterised by ESR. The semiquinone formation constant K is estimated to be 0.02 at pH 6-8, and

0.8 at pH 11-12 (Kano *et al.*, 1990a).

The pH dependence of the absorption spectra of the oxidized form of PQQ (PQQ_{ox}) has been interpreted with respect to pK_a values (Kano *et al.*, 1990a; 1990b). The pK_a^{ox} values were electrochemically determined to be 0.3, 1.6, 2.2, 3.3, and 10.3. They also estimated the pK_a^{red} values as follows: pK_{a,1}^{red} = 0.9, pK_{a,2}^{red} = 1.8, pK_{a,3}^{red} = 2.7, pK_{a,4}^{red} = 4.5, and pK_{a,5}^{red} = 8.5 (for the phenolic proton).

The acid-base equilibria of the reduced PQQ are more complex since there are dissociations of the phenolic protons in addition to those of the protonated pyridine nitrogen, the carboxyl groups, and the pyrrole proton.

The electrochemical properties of PQQ have been observed by cyclic voltammetry under acidic conditions using a di-(4-pyridyl) disulphide (4-pyds) modified gold electrode (Nakamura *et al.*, 1993). Electron transfer was reversible from pH 2 to 5, but not reversible above pH 5. The half-wave potential ($E_{1/2}$) and peak separation (ΔE_p) were calculated to be +144 mV and 37 mV respectively; the ΔE_p value indicated that the redox reaction occurred in a one-step two-electron transfer. Nakamura *et al.* (1993) suggested that PQQ interacts with the 4-pyds modified gold electrode to receive two electrons and two protons at the *o*-quinone moiety under acidic conditions. They proposed that the pyridine moiety of the 4-pyds modified electrode interacts with the *o*-quinone of PQQ via hydrogen bonds, which accelerate the proton transfer as well as the electron transfer. The indistinct, irreversible voltammetric response observed at

pH > 5 was considered to be due to the removal of a proton from the pyridine moiety in 4-pyds or the hydration of the *o*-quinone moiety of PQQ (Nakamura *et al.*, 1993).

1.6 ELECTROCHEMICAL OXIDATION AND REDUCTION OF PQQ USING A CONDUCTING POLYPYRROLE COATED ELECTRODE

Shinohara *et al.* (1991) observed that the electrochemical oxidation and reduction of PQQ using conventional electrodes (i.e. Pt, Au, or glassy carbon) are irreversible because of difficulties in the direct electron transfer between PQQ and these electrodes. Reversible oxidation and reduction of PQQ was subsequently achieved with a polypyrrole modified electrode (Shinohara *et al.* 1991). The corresponding redox potential was -110 mV (vs. Ag/AgCl) with a peak separation 40 mV. The peak separation proved that the redox reaction occurred in a one step two electron transfer process, and the peak current was proportional to the square root of scan rate which indicated that this PQQ-polypyrrole electron transfer system was diffusion controlled. The PQQ entrapped within the polypyrrole matrix retained its redox properties and the amount of PQQ entrapped was directly proportional to the thickness of the polymer membrane. Shinohara *et al.* concluded that the polypyrrole acted as a "molecular wire" between PQQ and the base electrode to facilitate reversible oxidation and reduction of the PQQ, and envisaged that the redox behaviour of PQQ in a conductive polymer matrix may be used to control the activity of quinoproteins electrochemically, (Shinohara *et al.*, 1991).

Detailed studies of the redox properties of PQQ aid our understanding of the complex

mechanisms of quinoprotein catalysis. Furthermore, characterisation and quantification of redox forms of PQQ is fundamental to the detection of quinoprotein electron transfer within biosensor configurations (Davidson, 1991).

1.7 ELECTRON TRANSFER OF QUINOPROTEINS - RELEVANCE FOR BIOSENSORS

The importance of the binding and electron transfer properties of PQQ within quinoproteins is critical to their successful incorporation in biosensors. The mode and specificity, of PQQ substrate binding will affect the type of substrate detected, the nature of the corresponding biochemical interaction, and the immobilisation of the quinoprotein.

Depending on the quinoprotein dehydrogenase, the natural electron acceptor is either a c-type cytochrome, a "blue" type copper protein (such as amicyanin in the case of MADH) or a membrane bound ubiquinone (Davidson and Jones, 1991). Hence quinoproteins normally donate electrons to a redox centre within a complex macromolecular matrix (Davidson, 1991). PQQ stimulated the oxidation of glucose to gluconate in membrane vesicles of *Eschericia coli* generating a proton motive force for uptake of lactose, alanine, and glutamate (van Schie *et al.*, 1985). Reconstitution of GDH with limiting amounts of PQQ allowed manipulation of the rate of electron transfer in membrane vesicles and whole cells (Ameyama *et al.*, 1987). Inter-molecular electron transfer was also implicated in the formation of semiquinone via reduced and oxidized cofactors of MADH (Davidson *et al.*, 1990). It was proposed that a common

domain of MADH may help to stabilize protein-protein interactions and facilitate both inter-molecular electron transfer between quinone cofactors and transfer to the copper centre of amicyanin (the natural electron acceptor for MADH).

It has now been established that long range inter-molecular electron transfer occurs between the redox centres of MADH and amicyanin (Davidson & Jones, 1991; Brooks *et al.*, 1994). Kinetic, physical, and structural data on these proteins indicate that a combination of electrostatic and hydrophobic interactions between amicyanin and MADH are involved in the proper orientation of the proteins to enable inter-molecular electron transfer. Understanding the mechanism of this long range electron transfer through quinoproteins is critical to the electrochemical detection of PQQ-mediated electron transfer in quinoprotein biosensors.

1.8 DIRECT BIOELECTROCATALYSIS OF QUINOPROTEINS

Burrows *et al.* (1991) recently observed the catalytic response of MADH at an edge plane graphite electrode (*epg*) and at a modified gold electrode in the absence of mediator. In both cases anodic waves were produced on addition of methylamine substrate. The authors concluded that the spatial arrangement of the C-O functionalities of the *epg* surface or the modifier molecules on the gold surface provide sufficient interaction with the positively charged subunit of MADH.

This research indicated that MADH interacts more specifically at the electrode surface than smaller redox proteins which could permit its application in development of non-

mediated quinoprotein enzyme electrodes. Whilst detection of methylamine has limited commercial application, detailed electrochemical analysis of this enzyme will assist the development of mediated and non-mediated quinoprotein biosensors for measurement of fructose, alcohol and glucose. Indeed, FDH has been widely used in a variety of colorimetric and flow injection analyses for the sensitive and specific determination of fructose (Ameyama, 1982; Nakashima *et al.*, 1985; Matsumoto *et al.*, 1986; 1988). More recent developments include the fabrication of several amperometric fructose sensors based upon direct electrochemistry of FDH in the absence of mediators (Khan *et al.*, 1991; Ikeda *et al.*, 1991).

In the first of these examples direct electron transfer between a monolayer of quinoprotein oxidoreductase, FDH and various electrodes such as Pt, Au and GC has been investigated (Khan *et al.*, 1991). The electron transfer between adsorbed FDH and the electrode proceeded directly and reversibly at all the electrodes. The metallic surface provided more rapid electron transfer with lower enzyme activity, whilst the carbon electrode produced slower electron transfer with higher dehydrogenase activity. Khan *et al.* concluded from their investigations that the electrochemical adsorption of FDH facilitated molecular orientation of FDH on the electrode surface, which shortened considerably the distance between the active site (PQQ) and the electrode surface, resulting in easier direct electron transfer (Khan *et al.*, 1991).

An alternative amperometric fructose sensor based on direct bioelectrocatalysis was described by Ikeda *et al.* in 1991. In this system an FDH modified carbon paste

electrode showed a current response to D-fructose in the absence of electron transfer mediators, and the current response was independent of the oxygen concentration in the solution. The maximum current response was obtained at approximately pH 4.5-5.0 which compared favourably with the optimum pH of FDH, pH 4.0, as determined by Ameyama *et al.* (1981) and the current was mainly controlled by the kinetics of the FDH reaction in the enzyme layer since diffusional resistance to substrate within this thin film FDH carbon paste electrode was negligible (Ikeda *et al.*, 1991).

1.9 MEDIATED QUINONE / QUINOPROTEIN BIOSENSORS AND ELECTROCATALYSIS

The electrocatalysis of quinone-bound electrodes has been compared to the oxidation of ascorbic acid. The electrochemical surface catalysis of NADH has been facilitated by a deliberate chemical modification of surface *o*-quinoidal groups covalently bound to a pyrolytic graphite electrode (Tse & Kuwana, 1978). Electrode fouling at high concentrations of NADH restricts the analytical use of such chemically modified enzyme electrodes (C.M.E.). The problems associated with NAD(P)⁺-dependent catalysis can be overcome by the use of PQQ containing quinoproteins which have no external cofactor requirement.

A biofuel cell incorporating the quinoprotein alcohol dehydrogenase (ADH) has provided a sensitive method for the detection of primary alcohols which required the presence of ammonium ions (Davis *et al.*, 1983). Quantitative kinetic data for the homogenous reaction between ADH and the mediator N,N,N',N'-tetramethyl-4-

phenylenediamine (TMPD) were obtained under conditions of substrate excess, in accordance with the Nicholson and Shain theory of catalytically coupled reversible electron transfer (Nicholson & Shain, 1964). This TMPD / ADH / methanol system remained catalytically coupled without substantial deterioration at pH 10.5 and 20° C for more than 20 days. Ten successive additions of 10 nmol methanol gave consistent results with a mean value of 3.47 Faraday mol⁻¹ and a standard deviation of 0.12 Faraday mol⁻¹ which illustrated the successful incorporation of ADH for accurate and reproducible alcohol detection (Davis *et al.*, 1983).

A subsequent enzyme electrode based on the coupling of MDH with TTF-TCNQ was reported by Zhao and Lennox in 1991. Oxidation of the reduced form of the enzyme at this organic conducting salt (OCS) electrode was achieved without ammonium ion activation, (Zhao & Lennox, 1991). This is in sharp contrast to the ammonium ion requirement of the *in vitro* assay of MDH in homogeneous solution. This increases the possibility of producing a practical biosensor for routine alcohol testing. However, in order to fully exploit the potential of this MDH / OCS electrode the equilibration time required to produce a low background current needs to be reduced significantly below the 6 hours reported by Zhao & Lennox.

D'Costa *et al.* (1985; 1986) developed a biosensor based on GDH which produced more than twice the current density of similar glucose oxidase (GOD) electrodes. The enzyme was immobilised onto graphite working electrodes with carbodiimide, and its electrochemistry determined with TMPD and ferrocene monocarboxylic acid (FMCA).

The temperature, pH stability and oxygen-independent response characteristics of this GDH based glucose sensor demonstrated its suitability for many practical applications. In particular, the high catalytic activity (GDH turnover number $320,000 \text{ min}^{-1}$), and oxygen insensitivity of this biosensor make it suitable for *in vivo* blood glucose monitoring in the management of diabetes, provided enzyme stability could be improved.

GDH has been incorporated into an enzyme electrode for blood glucose determination (Mullen *et al.* 1985, 1986). This DCPIP mediated enzyme electrode was hindered by 30 minute response times and the upper linear range was restricted to 0.25 mM. An alternative mediator PES produced a faster response and was linear up to 0.8 mM but this system was unstable at glucose concentrations $> 1.2 \text{ mM}$. The authors suggested that further investigation was required to optimise the coupling of suitable mediators within quinoprotein biosensors.

More recently, glucose electrodes were prepared using GDH immobilised on glassy carbon with an osmium complex containing a redox-conducting epoxy network (Ye *et al.*, 1993). The current density at 70 mM glucose reached $1.8 \mu\text{A cm}^{-2}$ when $15 \mu\text{g cm}^{-2}$ of the enzyme having an activity of $250 \text{ units mg}^{-1}$ was applied to the electrode. Under the same conditions, electrodes made with GOD of similar activity ($250 \text{ units mg}^{-1}$) had a maximum current density of $0.66 \mu\text{A cm}^{-2}$. The maximum current density was reached with 8 % GDH in the redox polymer film. The current density was almost linear through the 6.3 - 8.8 pH range and was not altered when the solution was either

aerated or argon purged. It decreased to half its initial value in 8 hours at 25°C.

A simplified fructose biosensor based on FDH with ferrocyanide measured amperometrically at +0.385 V was reported by Xie *et al.* in 1991. The sensor's response was characterised with respect to pH, specificity, stability and its application for determination of fructose in commercial fruit juices was demonstrated. The maximum current response occurred between pH 4.5 - 5.0, whilst a linear range was observed between 10 μ M and 1 mM fructose with a correlation coefficient of 0.999. The long term stability of this sensor, studied over a 5-month period, revealed that the electrode retained 90 % activity after 20 days, 75 % after 1 month, 60 % after 2 months and then remained constant at 60 % there after. The commercial application of this FDH sensor was evaluated by determination of fructose in various commercial fruit juices. The results gave an average correlation coefficient of 0.998 when compared with results using the standard AOAC method (Xie *et al.*, 1991).

1.10 PQQ - MODIFIED ENZYME ELECTRODES

The electrochemistry of PQQ was studied in solution and PQQ immobilized on a gold electrode modified with a chemisorbed cystamine monolayer (Katz *et al.*, 1994a). An electrochemically reversible diffusion-controlled reaction ($k = 1.7 \times 10^3 \text{ cm s}^{-1}$ at pH 7.0) was observed for PQQ in solution at the cystamine-modified electrode under acidic and neutral conditions ($\text{pH} \leq 7.0$) when the surface amino groups are positively charged. However, the electrochemical reduction of PQQ is completely irreversible on a non-modified gold electrode as well as on an electrode surface modified with neutral or

negatively charged groups. The cystamine monolayer on the gold electrode was used as a basis for the covalent immobilization of PQQ via carbodiimide coupling of the PQQ carboxylic groups with the surface amino groups. The electrochemical reaction of the immobilized PQQ was reversible over a wide pH range (pH 2-11). The PQQ modified electrodes exhibited very high stability.

A surface concentration of $1 \times 10^{-10} \text{ mol cm}^{-2}$, corresponding to a monolayer of PQQ, and an electron transfer rate constant k_s of 3.3 s^{-1} (pH 7.0) were determined for the PQQ-modified electrode. The total amount of immobilized PQQ could be increased dramatically if a gold electrode with a very high surface roughness was used. The redox potential E° of $-0.125 \text{ V} \pm 0.003 \text{ V}$ vs S.C.E. (pH 7.0) was obtained for both PQQ in solution and immobilized PQQ. It was suggested that the PQQ-modified electrodes developed could be used to prepare biosensors based on PQQ enzymes and to facilitate chemical reactions characteristic of PQQ directly on the electrode surface (Katz *et al.*, 1994a).

Reconstitution of GDH from its apoenzyme was also achieved on a gold electrode surface modified with a monolayer of PQQ (Katz *et al.*, 1994b). In this instance the gold electrode surface was modified with a chemisorbed monolayer of cystamine, and the cystamine amino groups were used for a covalent immobilization of PQQ. The spacer length between the electrode surface and the immobilized PQQ was increased using glutaraldehyde and 1,8-diaminooctane as additional spacers. Both kinds of PQQ modified electrodes (with short and long spacers) exhibited a reversible electrochemical

process with the redox potential -0.125 V (vs. S.C.E.) at pH 7.0.

The electrode modified with PQQ attached to long spacers was used as a support for the immobilization of GDH apoenzyme having affinity for PQQ which is its native cofactor. After the anchorage of the GDH apoenzyme, this electrode exhibited enzymatic activity for glucose oxidation. Electrocatalytic oxidation of glucose was obtained only in the presence of a soluble electron transfer mediator (DCPIP) which has a more positive redox potential than the immobilized PQQ. Therefore, although the enzymatic activity was reconstituted by the interaction of the apoenzyme binding site with the "long-spacered" PQQ immobilized as a monolayer on the electrode surface, there was no direct electron transfer between the electrode surface and the PQQ redox site inside the holoenzyme. Attempts to reconstitute the holoenzyme from the electrode surfaces modified with "short-spacered" PQQ as well as with the long spacers without PQQ resulted in electrodes without any catalytic activity (Katz *et al.*, 1994b).

Electrocatalytic oxidation of NADH and NADPH was subsequently shown by the same authors at PQQ modified electrodes. This process was strongly enhanced in the presence of Ca^{2+} cations. Cyclic voltammetry, steady-state current, rotating disk electrode and flow-injection measurements were applied to study the electrocatalytic oxidation (Katz *et al.*, 1994c). The kinetic parameters of this process were evaluated in the presence and absence of Ca^{2+} cations assuming the formation of an intermediate charge-transfer complex between the immobilized PQQ and NADH. The only influence of Ca^{2+} cations on the kinetics of the catalytic process is an equilibrium shift towards

the formation of the charge-transfer complex $[\text{NADH}^{\cdot\cdot}\text{Ca}^{2+}\text{PQQ}]$. The decay of this complex leading to the formation of the final products (NAD^+ and PQQH_2) was found to be independent of the presence of Ca^{2+} cations. The process studied was considered as a model for the mechanism of catalysis exhibited by PQQ- and Ca^{2+} -containing dehydrogenases. These PQQ-modified electrodes were stable enough even in a flow-injection system and could be very promising for future practical applications (Katz *et al.*, 1994c).

Willner & Riklin (1994) employed similar technology to develop an amperometric sensor utilising the NAD(P)^+ -cofactor-dependent enzyme, malic dehydrogenase, at a PQQ-enzyme monolayer-modified electrode. The resulting PQQ-monolayer electrode (PQQ surface coverage $1.98 \times 10^{-10} \text{ mol.cm}^{-2}$) catalysed the electro-oxidation of NADPH and NADH. Corresponding anodic currents, controlled by the concentrations of NADH and NADPH respectively, provided an amperometric sensor for the cofactor. Malic dehydrogenase was covalently linked to the PQQ-monolayer electrode and the resultant PQQ-enzyme electrode (enzyme coverage $4.01 \times 10^{-12} \text{ mol.cm}^{-2}$) facilitated amperometric determination of malic acid in the presence of the cofactor NADP^+ . In this system, biocatalytic oxidation of malic acid generated NADPH which was oxidized by the PQQ component back to NADP (Willner & Riklin 1994). It is envisaged that the methodology applied by Katz *et al.* (1994 a,b,c.) and Willner & Riklin (1994) should permit future development of biosensors utilising other quinoproteins and/or PQQ.

1.11 AIMS OF PROJECT

The objective of this research was to examine the development of quinoprotein dehydrogenase biosensors and pyrroloquinoline quinone (PQQ) modified enzyme electrodes. Three approaches were taken:

The first objective of the work, was to determine the enzyme stability and to confirm the lack of ammonium ion requirement of quinoprotein methanol dehydrogenase at a TTF.TCNQ organic conducting salt electrode. The ability to replace the *in vitro* ammonium ion requirement of methanol dehydrogenase with a TTF.TCNQ modified electrode will enable construction of more practical sensors for methanol without external addition of ammonium ions which complicates current MDH sensors.

The second objective, was to examine quinoprotein methylamine dehydrogenase in a mediated enzyme electrode. The aim of this work was to detect histamine amperometrically without the need for extensive sample pretreatments currently required in HPLC and GLC analysis. The incorporation of MADH into an enzyme electrode will provide further confirmation that NAD(P)⁺-independent quinoprotein dehydrogenases are well suited for biosensor development.

The final objective, was to investigate the use of PQQ as a mediator for biosensor development. The low redox potential of PQQ observed at a polypyrrole-modified electrode, (-110 mV vs. SCE, Shinohara *et al.*, 1991), may reduce/remove possible interference from other electroactive species such as ascorbic acid. Furthermore,

since PQQ has already shown reversible electron transfer when coupled to NAD-dependent enzymes, the electrochemical coupling of PQQ with the FAD-dependent glucose oxidase merits investigation. These investigations would confirm that PQQ mediated electron transfer is not restricted to a single class of enzymes and thereby increase the possibility of using PQQ as an alternative mediator for widespread biosensor development.

2. AMMONIUM ION REQUIREMENT AND STABILITY OF METHANOL DEHYDROGENASE TTF.TCNQ ELECTRODES

2.1 INTRODUCTION

Although methanol dehydrogenase was first characterised in 1964 (Anthony & Zatman, 1964) from *Pseudomonas* sp. M27 and subsequently purified from several sources, there is still confusion in the literature regarding the steady-state kinetic properties of this enzyme and disagreement as to its reaction mechanism (Anthony, 1993). The study of this enzyme is complicated by the fact that the enzyme catalyzes the reduction of an electron acceptor in the absence of any added substrate (Anthony & Zatman, 1964; Duine & Frank, 1980; Ghosh & Quayle, 1981). This endogenous rate is not significantly stimulated by the addition of substrate. Furthermore, if allowed to react in this manner, the enzyme becomes inactivated. The commonly accepted explanation for this substrate-independent activity is that the enzyme or components of its assay are contaminated by an unidentified endogenous substrate. This endogenous activity is suppressed by cyanide, which also protects the enzyme against inactivation (Duine & Frank, 1980). NAD(P)⁺-independent methanol dehydrogenases linked to artificial electron acceptors *in vitro* have a requirement for ammonium ions or primary amines as activators. *In vivo*, methanol dehydrogenase is coupled to cytochrome *c*_L, a class of *c*-type cytochrome which is distinctive in having a low isoelectric point (pI = 3-4), large size (17-kDa), and little sequence similarity to other bacterial or eukaryotic *c*-type cytochromes (Anthony, 1992b). The enzyme is located on the periplasmic side of the cytoplasmic membrane in *Methylophilus methylotrophus* (Jones *et al.*, 1982) and *Paracoccus denitrificans* (Alefounder & Ferguson, 1981). Evidence that methanol

dehydrogenase contained a novel nitrogen containing quinone coenzyme was obtained by electron spin resonance spectroscopy (Duine *et al.*, 1978; DeBeer *et al.*, 1979; Westerling *et al.*, 1979; Duine & Frank, 1980). Confirmation of the quinone structure was obtained by X-ray crystallography (Salisbury *et al.*, 1979), nuclear magnetic resonance spectroscopy and mass spectrometry of the purified coenzyme (Duine *et al.*, 1980).

2.1.1 Polyelectrolytes and enzyme stability

A major disadvantage of many enzymes is that they are very labile in nature and require careful handling to avoid inactivation. Many techniques have been employed to increase the stability of enzymes including covalent immobilisation (Sundaram, 1982), entrapment (Martinek *et al.*, 1977), chemical modification (Marshall, 1978), crosslinking (Torchilin & Martinek 1979), protein engineering (Ahern *et al.*, 1987) and chemical additives (Ye *et al.* 1988).

Using combinations of the polyelectrolytes Gafquat 755-N and DEAE dextran as soluble additives to enzyme solutions Gibson *et al.* (1992a,b) have shown that it is possible to enhance the activity retention of enzymes in solution and during desiccation at ambient temperatures and to increase their thermal stability when tested under conditions of thermal stress. Gafquat 755N is wholly synthetic, being formed from vinylpyrrolidone and dimethylaminoethyl methacrylate reacted together to give a copolymer with pendant quaternary (positively charged) amino groups with a pK_a of about 14. The polymer is always ionised at pH values under 14 and with the high proportion of quaternary amino

groups present in Gafquat 755-N this means that the polyelectrolyte is a strong cation.

The structure of Gafquat is shown in Figure 2.1. This is a synthetic quaternary polymer of molecular weight about 1 000 000. In contrast Gantrez S-95, a hydrolysed copolymer of methyl vinyl ether and maleic acid (Figure 2.2), contains no quaternary amino groups, and is an anionic polymer whose negative charge is conferred by the maleic acid groups.

Gibson *et al.* (1992b) explained the stabilisation effect observed when enzymes are incubated in solution in the presence of polyelectrolytes. They proposed that the increased stability observed was due to the formation of soluble electrostatic protein-polymer complexes. The presence of polyelectrolyte enhanced the enzyme activity indicating a positive effect on enzyme stability. The electrostatic charge of the polyelectrolyte is important for enzyme stability. Polyelectrolytes associate with molecules of the opposite charge when in aqueous solution and in some cases precipitation occurs. These aggregates redissolve as the ionic strength is raised, but it is believed that the enzyme-polyelectrolyte interaction still occurs even at high ionic strength. The protein molecule at pH values below its isoelectric point (pI) will be positively charged overall and as such will attract and interact electrostatically with negatively charged ions. This can be shown to occur since solutions of proteins and polyions will flocculate under conditions of low ionic strength when mixed in the correct proportions (Gurov *et al.*, 1981; McKernan & Ricketts, 1960). For the stabilization of protein structure in aqueous solutions the type of protein, the

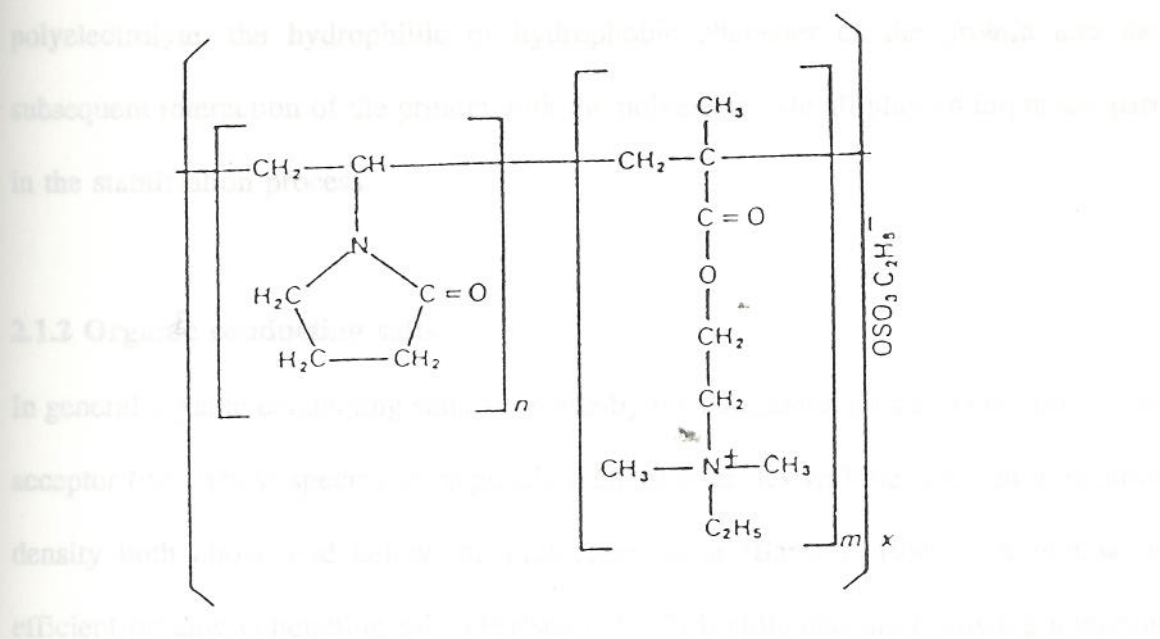


Figure 2.1. The structure of Gafquat 755-N a cationic polyelectrolyte composed of vinylpyrrolidone and dimethylamino methacrylate.

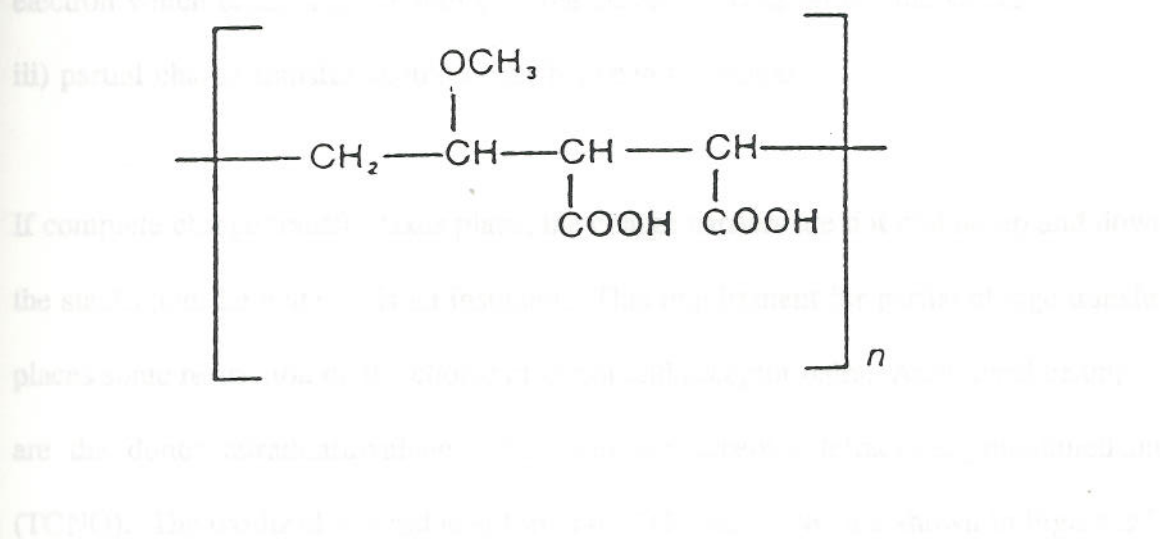


Figure 2.2. The structure of Gantrez S-95 an anionic polyelectrolyte composed of methylvinylether and maleic acid.

polyelectrolyte, the hydrophilic or hydrophobic character of the protein and the subsequent interaction of the protein with the polyelectrolyte all play an important part in the stabilization process.

2.1.2 Organic conducting salts

In general organic conducting salts are made by the combination of a donor (D) and an acceptor (A). These species are typically planar molecules with delocalized π -electron density both above and below the molecular plane (Bartlett, 1990). A review of efficient organic conducting salts (Perlstein, 1977) highlighted the following common features:

- i) A segregated stack structure in which all the donor molecules go into one type of stack and all the acceptor molecules into another;
- ii) the donor (or acceptor) form a new aromatic sextet by the loss (or gain) of an electron which ensures the mobility of the charge carriers within the stacks;
- iii) partial charge transfer should occur between the stacks.

If complete charge transfer takes place, the charge carriers are not mobile up and down the stacks and the material is an insulator. This requirement for partial charge transfer places some restriction on the choice of donor and acceptor pairs. Archetypal examples are the donor tetrathiafulvalene (TTF) and the acceptor tetracyanoquinodimethane (TCNQ). The oxidized and reduced forms of TTF and TCNQ are shown in Figure 2.3:

2.3.3 Electrochemical modification of organic conductive polymers

There has been considerable interest in whether the polymer is generated by a new electrochemical reaction or by a modification of the polymer already present. Experiments of this nature have been carried out with the polymer TTF. The polymer TTF is a conjugated polymer with a positive charge delocalized over the polymer chain. The polymer TTF is a conjugated polymer with a positive charge delocalized over the polymer chain. The polymer TTF is a conjugated polymer with a positive charge delocalized over the polymer chain.

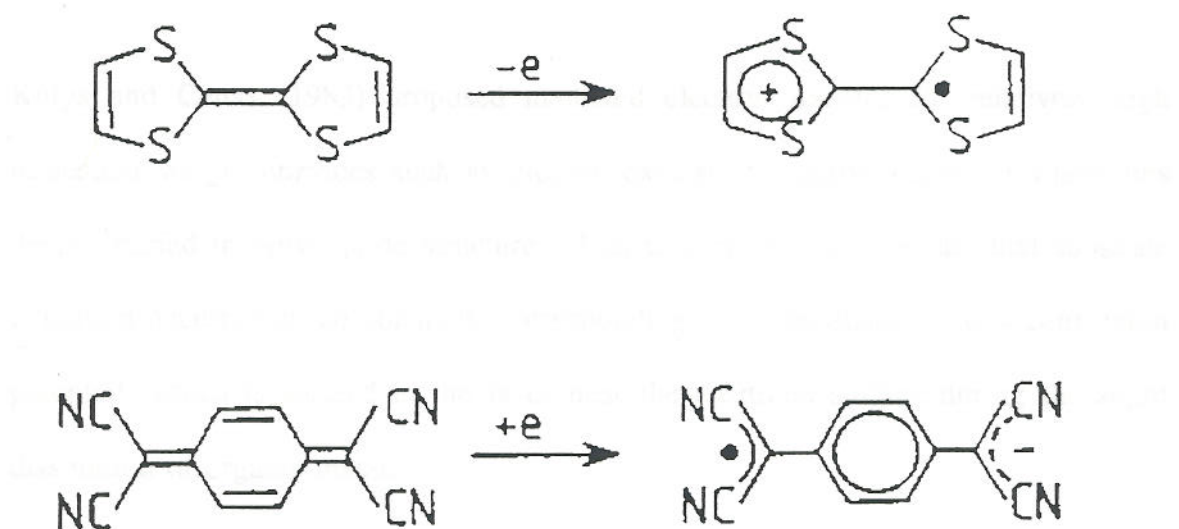


Figure 2.3. Structures of the oxidized and reduced forms of TTF and TCNQ

In both cases a new aromatic sextet is formed. As a consequence both the TTF and TCNQ stacks contribute to the conductivity in TTF.TCNQ organic salts (Bartlett, 1990).

2.1.3 Electrochemical mechanism of organic conducting salts

There has been some controversy as to whether the enzyme is oxidized by direct electron transfer on the surface of the organic conducting salt or whether mediated electron transfer takes place. Experimental and theoretical reports indicate three possible mechanisms for electron transfer between the redox centre of the enzyme and the surface of the organic conducting salt electrode. These are : i) homogeneous mediation, ii) direct electron transfer and iii) heterogeneous redox catalysis.

Kulys and Cenas (1983) proposed mediated electron transfer for relatively high molecular weight enzymes such as glucose oxidase, the active centre of which lies deeply buried in polypeptide structure. This is supported by the fact that substrate oxidation occurred at the potentials corresponding to the mediators' redox conversion potential, which is formed in the layer near the electrode surface during the slight dissolution of organic metal.

The extremely efficient oxidizing ability of the components of the organic metals TCNQ and TTF for the reduced enzyme has been illustrated (Kulys & Cenas, 1983; Hendry & Turner, 1988; Turner *et al.*, 1987a,b). These observations provide further support for a mediated mechanism of electron transfer between the redox centre of the enzyme and the organic-metal electrode.

However, Bartlett *et al.* (1985) disagree with the mediated mechanism and propose direct electron transfer between the enzyme and the surface of the organic conducting

salt. Experimental evidence for the direct electron transfer comes from three sources: determination of the rate of flux, scanning electron microscopy of the surface of a TTF.TCNQ electrode and insertion of a dialysis membrane between electrode and the enzyme, which inhibits activity completely (Bartlett *et al.*, 1985; Albery *et al.*, 1987). For all these reasons Albery *et al.* concluded that the reaction does not take place by homogeneous mediation as suggested by Kulys and Cenas (1983) but that the enzyme does react on the surface of the electrode.

It was subsequently suggested that the reaction of the enzyme on the organic conducting salt electrode occurred via heterogeneous redox catalysis as opposed to either direct or homogeneous mediated electron transfer (Albery *et al.*, 1987). This suggestion was based on the determination of reaction order with respect to enzyme. In the case of direct electron transfer the reaction order should be unity, but was in fact determined to be 0.5. which indicates that electron transport takes place by heterogeneous redox catalysis. Further evidence for heterogeneous redox catalysis was obtained from experiments showing the dependence of anodic current on the square root of enzyme concentration (Albery *et al.*, 1987).

Hill *et al.* (1988) supported the conclusion of Albery *et al.*, (1987) with results obtained from radio labelling experiments, they suggested that when a TTF.TCNQ electrode is soaked in enzyme at least two types of adsorbed layers are formed at the interface. The first layer is irreversibly adsorbed and inactive, whereas the second one is weakly or reversibly adsorbed and is responsible for heterogeneous redox catalysis (Hill *et al.*,

1988).

2.2 MATERIALS AND METHODS

2.2.1 Reagents

All reagents (phenazine ethosulphate (PES); 2,6-dichlorophenolindophenol (DCPIP) ammonium chloride; sodium phosphate and Tris buffer) were supplied by Sigma/Aldrich (Dorset, U.K.) or BDH (BDH Ltd., Poole) and were of analytical reagent grade. The polyelectrolytes Gafquat 755-N and Gantrez S-95 were kindly supplied by Dr. Tim Gibson (Leeds Biochemicals, Leeds, U.K.).

The buffer used for investigation of MDH stability in solution was 0.1 M Tris-HCl, pH 9.0. Electrochemical experiments involving MDH-TTF.TCNQ packed cavity electrodes were performed using 0.2 M phosphate buffer, pH 8.0 containing 0.1 M KCl and 50 mM ammonium chloride unless otherwise stated. Spectroscopic grade methanol was supplied by Fisons (Loughborough, Leics.) and all water was purified by reverse osmosis.

2.2.2 Apparatus

Cyclic voltammetry and amperometry experiments were performed using an Autolab Electrochemical Analyser (Windsor Scientific, Windsor) interfaced to a Viglen computer (Viglen Ltd., London) and an Epson LX-400 printer (Epson Ltd., Telford). The voltammograms were produced by importing data from the Autolab software (GPES 3, Ecochemie, Netherlands) into a graphics package (Sigmaplot, Jandel

Corporation, USA). A saturated calomel electrode (S.C.E.) supplied by Russell (Auchtermuchty, Fife) was used as a reference and the auxiliary electrode was a 1 mm diameter platinum wire (BDH, Merck Limited, Poole, Dorset).

2.2.3 Source of quinoprotein methanol dehydrogenase

Methanol dehydrogenase from *Methylophilus methylotrophus* (Sigma, Poole, Dorset.) supplied as freeze dried crystals was stored at -80°C until required. Enzyme isolated from *Paracoccus denitrificans* (Davidson *et al.*, 1989) was supplied frozen in 50 mM potassium phosphate, pH 7.5, containing 10 % ethylene glycol to maintain long term storage. Unless otherwise stated, for investigations of methanol dehydrogenase stability in solution, 1.6 mg.ml⁻¹ solutions of MDH from both bacterial sources were prepared in 0.1 M Tris-HCl buffer pH 9.0 and kept at 4°C until used. MDH-TTF.TCNQ packed cavity electrodes were prepared with separate 2 mg aliquots of enzyme from *Methylophilus methylotrophus* and *Paracoccus denitrificans* respectively.

2.2.4 Stability of quinoprotein methanol dehydrogenase

Reactions were carried out in a spectrophotometer cuvette at 30°C containing: 0.1 M Tris buffer, pH 9.0, 0.167 ml; 0.45 M ammonium chloride, 0.033 ml; 2.6 mM DCPIP, 0.033 ml; 0.3 M methanol 0.033 ml, and 25 µl of MDH. The final volume was made to 1 ml with distilled water. The reaction was initiated by the addition of 33 mM PES, 0.033 ml; and the decrease in absorption at 600 nm was measured against a reference cuvette containing distilled water. For determination of relative activity of MDH in solution a TecamTM Dri-Block heating mantle (Techne Ltd., Duxford,

Cambridge) was used to maintain enzyme solutions at 45°C, 50°C and 55°C respectively.

2.2.5 Effect of polyelectrolytes on enzyme stability

Aliquots of enzyme solution (1.6 mg.ml⁻¹) were mixed with 0.1 M Tris-HCl buffer pH 9.0 and a combination of the stabilizer (Gafquat 755-N and Gantrez S-97 respectively) to give a final concentration of 0.5 % stabilizer in a volume of 2 ml. Unstabilised enzyme preparations contained buffer and enzyme solution only. Control experiments containing polyelectrolytes together with all other assay components except enzyme established that the polyelectrolytes were not responsible for any decrease in absorbance at 600nm.

2.2.6 Preparation of TTF.TCNQ organic conducting salt

Separate 0.45 g portions of TTF (Aldrich Chemical Co., Gillingham, Dorset) and TCNQ (Sigma, Poole, Dorset) were dissolved in corresponding 25 ml volumes of hot spectroscopic grade acetonitrile (Fisons, Loughborough, Leics). This was carried out in a fume cupboard using round bottomed flasks clamped into a heating mantle. Once dissolved, both solutions were then mixed under reflux to produce a black precipitate. This mixture was cooled overnight in a stoppered flask with constant stirring. The resultant black powder was filtered under vacuum using a solvent resistant filter pump and washed with cold spectroscopic grade acetonitrile. Washing was repeated with diethyl ether until the filtrate produced was colourless. The TTF.TCNQ powder was finally dried in a sealed desiccator jar (at room temperature) for a minimum of 12 hours

until a constant weight was achieved, (Cass, 1990). The conducting salt itself is very stable and can be kept at room temperature for more than 2 years. It is important to use spectroscopic grade acetonitrile in the preparation since other grades of acetonitrile contain impurities (such as acrylonitrile) which lead to adverse side reactions (Cass 1990).

Prior to accepting TTF.TCNQ for use within the packed cavity electrode, the electrochemical stability of the organic conducting salt was determined. Samples exhibiting peaks in cyclic voltammograms between +350 and -200 mV vs Ag/AgCl were not used for enzyme electrochemistry.

2.2.7 Preparation of MDH-TTF.TCNQ packed cavity electrodes

A schematic diagram of the packed cavity electrode is shown in Figure 2.4. The electrode consisted of a platinum wire (1.0 mm diameter) recessed into a PTFE casing. High temperature silicon oil (Aldrich, Dorset, U.K.) was added to dry TTF.TCNQ (1:10 v/v) and mixed thoroughly before application. The use of silicon oil (poly(methylphenylsiloxane)) provides reproducible working electrodes which avoids the problems encountered with dispersing TTF.TCNQ in a poly(vinyl chloride) (PVC) or polystyrene (PS) matrix (Zhao & Lennox, 1991).

The TTF.TCNQ/silicon oil slurry was compressed into a cavity (depth 0.5 mm) and MDH from either *Methylophilus methylotrophus* (Sigma, Poole, Dorset.) or enzyme isolated from *Paracoccus denitrificans* (Davidson *et al.*, 1989) was placed on the

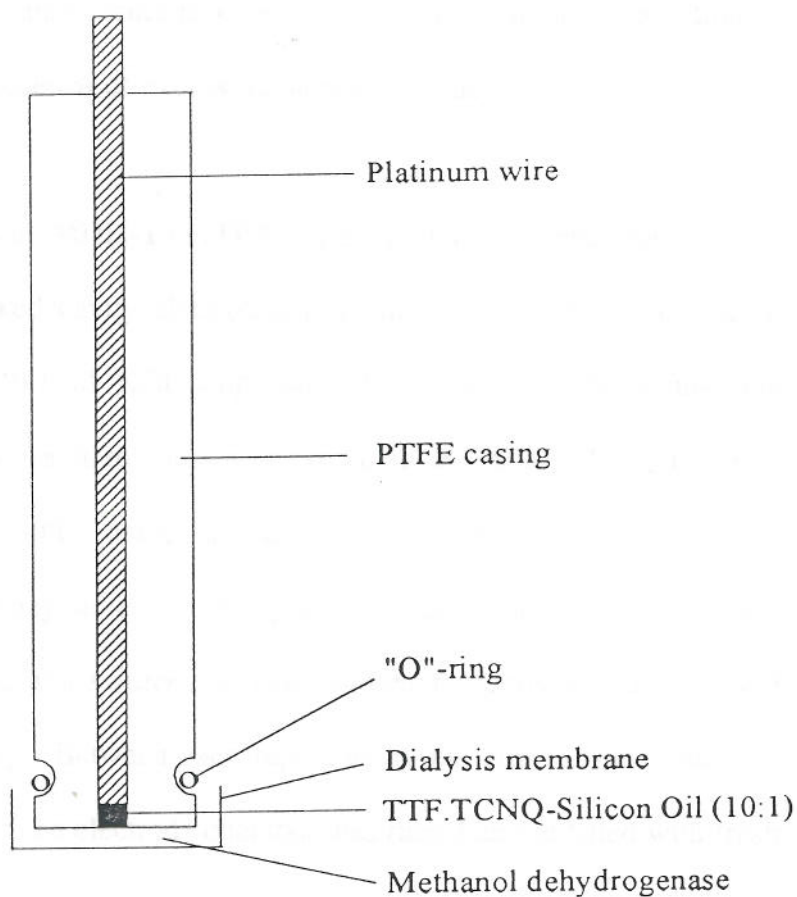


Figure 2.4. Schematic diagram of a TTF.TCNQ-MDH packed cavity electrode. The electrode consisted of a platinum wire (1.0 mm diameter) recessed into a PTFE casing. The TTF.TCNQ was compressed into a cavity (depth 0.5 mm) and MDH was retained behind a dialysis membrane held in place by rubber "O" rings.

TTF.TCNQ electrode surface followed by 1 μ l of potassium phosphate buffer (0.20 M, pH 8.0). The MDH-TTF.TCNQ packed cavity electrode was then covered with 1 cm² Spectra/PorTM dialysis membrane held in place with a 12 x 2 mm "O" ring.

Unless indicated otherwise, all MDH-TTF.TCNQ packed cavity electrodes were poised at +200 mV vs. S.C.E. for amperometric detection of current upon the addition of methanol substrate in the presence of excess ammonium ion activator (50 mM).

2.2.8 Operational stability of MDH-TTF.TCNQ packed cavity electrodes

The MDH-TTF.TCNQ packed cavity electrodes were immersed in 10 ml of 0.2 M phosphate buffer, pH 8.0 (0.1 M KCl supporting electrolyte, 50 mM ammonium chloride activator) contained in a 20 ml glass water-jacketed cell (Soham Scientific, Ely, Cambs.) thermostated at 30°C using a circulating water bath (Gallenkamp, Loughborough, Leics.). Enzyme electrodes prepared using MDH isolated from *Methylophilus methlotrophus* and *Paracoccus denitrificans* respectively were assayed at least four times repeatedly. Between each repeat assay the enzyme electrode was stored for 5 min at 4°C whilst the electrode chamber was rinsed and re-filled with fresh assay buffer.

2.2.9 Thermal stability of MDH-TTF.TCNQ packed cavity electrodes

For temperature profile experiments the circulating water bath was used to maintain the temperature at 30°C, 35°C, 40°C, 45°C and 50°C, respectively. Enzyme electrodes prepared using MDH isolated from *Paracoccus denitrificans* were initially assayed at

each of the different temperatures stated above (time zero), and then re-assayed after 60, 90, 120 and 150 min respectively.

2.2.10 Effect of ammonium ions on MDH-TTF.TCNQ packed cavity electrodes

During investigation of the effect of ammonium ions the phosphate buffer (0.2 M, pH 8.0) contained saturating levels of methanol substrate (2 mM), and the ammonium ion concentration was varied between 0 and 50 mM. Reciprocal plots of the amperometric responses obtained using packed cavity electrodes prepared with enzyme isolated from *Methylophilus methylotrophus* and *Paracoccus denitrificans* were used to determine the apparent kinetic parameters of each enzyme electrode with respect to ammonium ion concentration.

Similar reciprocal plots, derived from the amperometric response obtained at excess ammonium ion concentration, were used to determine the apparent kinetic parameters of each enzyme electrode with respect to methanol substrate concentration.

2.3 RESULTS AND DISCUSSION

2.3.1 Relative activity of methanol dehydrogenase isolated from *Methylophilus methylotrophus*

The relative activity of MDH (*Methylophilus methylotrophus*) incubated at 45°C is shown in Figure 2.5. The results indicate that the relative activity of this enzyme is 23 % after 20 min, falling to < 6 % after 40 min. It is evident that MDH isolated from *Methylophilus methylotrophus* is not stable in solution when incubated at 45°C.

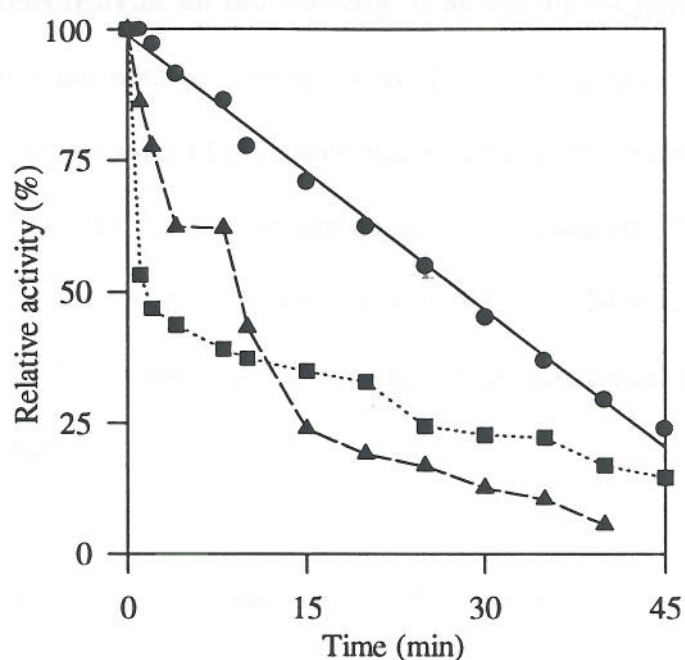


Figure 2.5. Relative activity of 1.6 mg.ml^{-1} methanol dehydrogenase isolated from *Methylophilus methylotrophus* incubated at 45°C : —▲— MDH-no polyelectrolyte ; —■— MDH-Gafquat 755-N(0.5%), a cationic polyelectrolyte; —●— MDH-Gantrez 95-N(0.5%), an anionic polyelectrolyte.

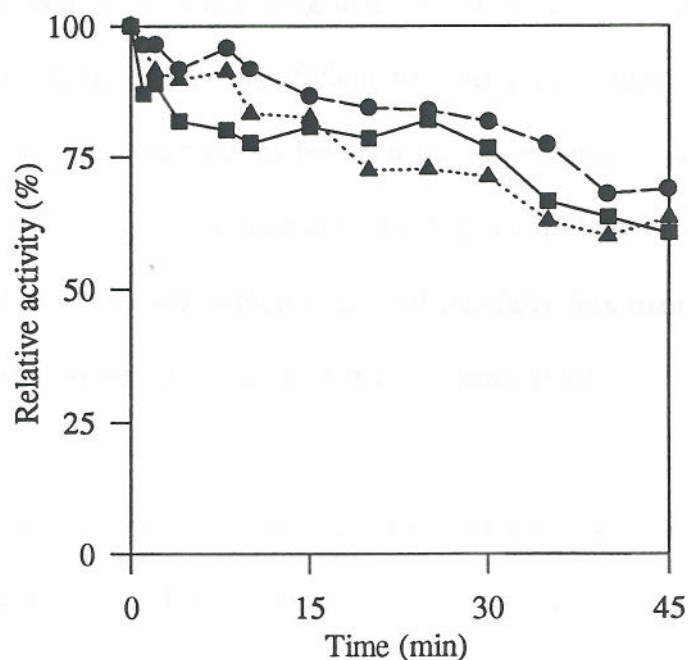


Figure 2.6. Relative activity of 1.6 mg.ml^{-1} methanol dehydrogenase isolated from *Paracoccus denitrificans* incubated without polyelectrolytes at —●— 45°C ; —■— 50°C ; —▲— 55°C . Activity determined with respect to $\mu\text{moles DCPIP}$ reduced per min at 600 nm , where 100 % relative activity represents MDH activity at time zero.

2.3.2 Effect of polyelectrolytes on the stability of methanol dehydrogenase

Figure 2.5 also shows the relative activity of MDH (*Methylophilus methylotrophus*) incubated at 45°C in the presence of polyelectrolytes Gafquat 755-N and Gantrez S-95. The relative activity of the enzyme in the presence of Gafquat 755-N a cationic polyelectrolyte (48 % after 5 min, 36 % after 20 min, falling to 20 % after 40 min) was not very different from the relative activity of methanol dehydrogenase incubated in the absence of polyelectrolytes.

The stability of methanol dehydrogenase isolated from *Methylophilus methylotrophus* was increased at 45°C in the presence of the anionic polyelectrolyte Gantrez S-95 (relative activity 96 % after 5 min, 62 % after 20 min, falling to 38 % after 40 min). Whilst incubation with Gantrez polyelectrolyte preserved 62 % of the enzyme activity after 20 min incubation at 45°C, suggesting that some interaction occurs between the anionic Gantrez and MDH, this is insufficient to confer prolonged stability. It is possible that the electrostatic interactions between the polyelectrolyte and enzyme are insufficient in strength and number to enhance stability due to lack of positive charge on the surface of MDH at pH 9.0, which is not substantially less than the isoelectric point of MDH, reported to be 10.3 (Aston, 1984; Gossain, 1989).

When the enzyme was incubated with Gafquat 755-N no stability was conferred, as at pH 9.0 this cationic polymer did not interact with the slightly positively charged enzyme. This also indicates that the electrostatic interaction necessary for stability to be conferred in solution is dictated by the relative charge of the polymer and the

enzyme. These results further emphasise the extremely labile nature of MDH isolated from *Methylophilus methylotrophus* as indicated by previous studies of the storage stability of methanol dehydrogenase purified from *Methylophilus methylotrophus* (Gossain, 1989). Virtually all enzyme activity was lost within forty-eight hours when stored in the absence of methanol at room temperature, 4°C or -20°C. With the inclusion of 15 mM methanol in the buffer MDH retained approximately 70 % activity when stored at 4°C over a period of three weeks (Gossain, 1989).

2.3.3 Relative activity of methanol dehydrogenase isolated from *Paracoccus denitrificans*

The relative activity of methanol dehydrogenase (*Paracoccus denitrificans*) incubated in solution, without any polyelectrolyte, at 45°C, 50°C and 55°C is shown in Figure 2.6. After 40 min incubation at 45°C, 74 % activity remained ; at 50°C, 65 % activity remained and at 55°C the relative activity was 62 % after 40 min.

The enhanced thermal stability observed with MDH isolated from *Paracoccus denitrificans* is in good agreement with previous research by Davidson *et al.* (1992) who also observed that this enzyme was relatively resistant to thermal denaturation, retaining almost 100 % activity after a 10 min incubation at 60°C. A 35 % loss in activity was observed after 10 min incubation at 65°C and total loss of activity occurred after 10 min incubation at 70°C.

The results presented in Figures 2.5 & 2.6 indicate that the enzyme isolated from

Paracoccus denitrificans is much more stable than methanol dehydrogenase isolated from *Methylophilus methylotrophus*. Analysis of the known three dimensional structure of MDH from *Paracoccus denitrificans* (Chen *et al.*, 1992) and comparison with X-ray diffraction data for MDH from *Methylophilus methylotrophus* (Xia *et al.*, 1992), reveals no difference in the active site structure of both enzymes.

However, the isoelectric point of MDH from *Methylophilus methylotrophus* is 10.3 (Aston, 1984; Gossain 1989), whereas MDH from *Paracoccus denitrificans*, an acidic protein, has an isoelectric point of 3.7 (Bamforth & Quayle, 1978). Therefore, when assayed at pH 9.0, MDH isolated from *Paracoccus denitrificans* has a predominant negative charge which may contribute to the enhanced thermal stability observed for this enzyme.

The MDH isolated from *Paracoccus denitrificans* (Molecular weight 151,000; Ghosh & Quayle 1978) is larger than the enzyme isolated from *Methylophilus methylotrophus*, molecular weight 130,000 (Gossain, 1989). This difference in size between the two enzymes may also contribute to the increased thermal stability of MDH from *Paracoccus denitrificans* since more energy would be required to denature the larger protein.

The different stability observed in Figures 2.5 & 2.6 could also be attributed to the fact that both enzymes were supplied in different forms (*Methylophilus methylotrophus* MDH supplied as freeze dried crystals, *Paracoccus denitrificans* MDH supplied in

solution).

Whilst the amino acid sequences for the H and L subunits of MDH from *Paracoccus denitrificans* have been determined (Harms *et al.*, 1987; Van Spanning *et al.*, 1991), the amino acid sequences of MDH from *Methylophilus methylotrophus* are unknown. Future DNA sequencing of the latter enzyme may reveal sequence differences which could help to explain the different properties of the two MDHs.

2.3.4. The TTF.TCNQ organic conducting salt

2.3.4.1 Electrochemical stability of the TTF.TCNQ organic conducting salt

The voltammetric response observed at an organic conducting salt electrode depends on the sweep rate, choice of potential limits, the composition of the solution and the previous history of the electrode. A typical cyclic voltammogram for a clean TTF.TCNQ packed cavity electrode recorded in pH 8.0 buffer solution (operating pH of MDH-TTF.TCNQ electrode) is shown in Figure 2.7. This figure shows that the cyclic voltammogram is featureless over the stable potential range of the TTF.TCNQ organic conducting salt, (i.e. -0.2 V $\rightarrow$ +0.4 V), vs. S.C.E., scan rate 5 mV.s⁻¹.

2.3.4.2 Instability of organic conducting salt cycled beyond -0.2 and +0.4 volts

A typical cyclic voltammogram for TTF.TCNQ electrodes which have been cycled beyond the stable range in 0.2 M phosphate buffer pH 8.0, containing potassium chloride as the background electrolyte is shown in Figure 2.8. There are two distinct peaks in the cyclic voltammogram which correspond to surface transformations of the

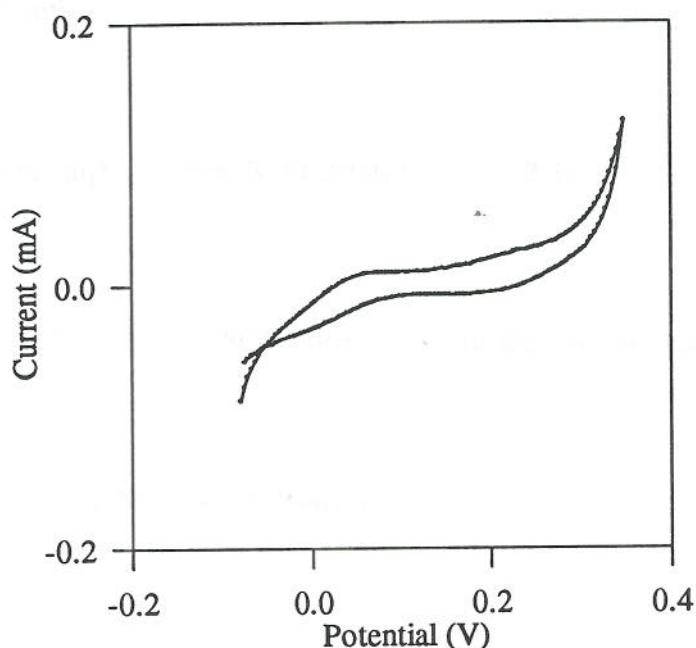


Figure 2.7. A typical cyclic voltammogram for clean TTF.TCNQ packed cavity electrodes recorded in 0.2 M phosphate buffer, pH 8.0, 0.1 M KCl background electrolyte. The cyclic voltammogram is featureless over the stable potential range of the TTF.TCNQ organic conducting salt, (i.e. -0.2 V $\rightarrow$ +0.4 V), scan rate 5 mV.s⁻¹.

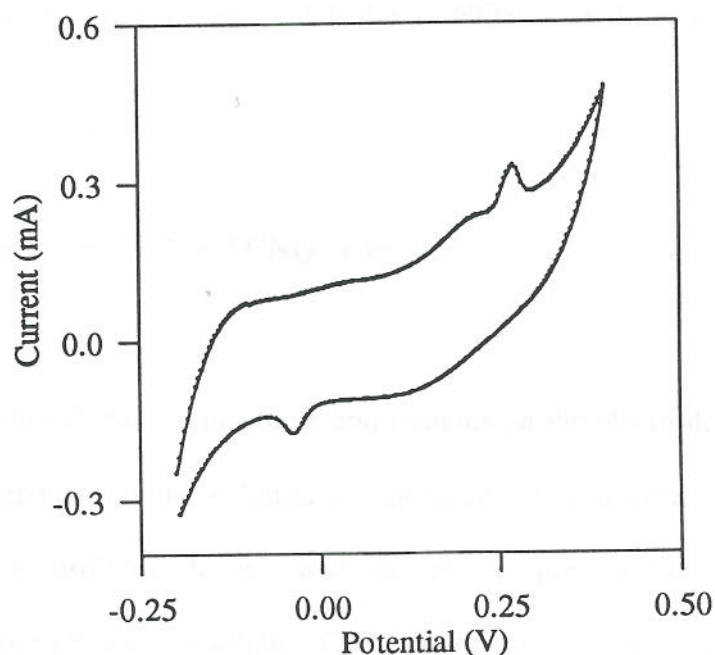
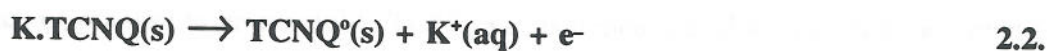


Figure 2.8. A typical cyclic voltammogram for TTF.TCNQ packed cavity electrodes cycled beyond the stable range in 0.2 M phosphate buffer pH 8.0, 0.1 M KCl background electrolyte. The cathodic peak at -0.05 V vs. S.C.E., and the anodic peak at +0.27 V correspond to surface transformations of the TTF.TCNQ organic conducting salt, scan rate 5 mV.s⁻¹

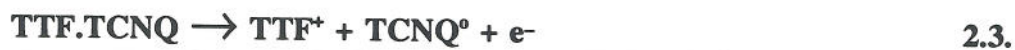
TTF.TCNQ organic conducting salt. The cathodic peak at -0.05 V versus S.C.E. corresponds to the reaction:



The anodic peak at +0.27 V versus SCE corresponds to the reverse reaction



The stable range is set by the decomposition of the electrode by either oxidation or reduction of the electrode constituents. If the TTF.TCNQ electrode is taken to potentials more positive than about +0.4 V versus SCE the TCNQ° is oxidized back to neutral TCNQ as illustrated in equation 2.3.



In aqueous solution the TCNQ° is insoluble and remains on the electrode surface. The fate of the TTF^+ depends on the solution composition. It can either dissolve into solution or form an insoluble deposit with the anions present in solution. For electrolytes containing chloride, insoluble TTF salts deposit on the electrode. These insoluble surface species are electroactive and show up during cyclic voltammetry.

If the clean electrode surface is taken to potentials more negative than about - 0.2 V versus SCE the TTF.TCNQ becomes reduced giving insoluble TTF⁰ and TCNQ⁻ as shown in equation 2.4.



This time the fate of the TCNQ⁻ depends on the composition of the electrolyte, in the presence of potassium an insoluble TCNQ salt is deposited which is electroactive at the electrode surface. Jaeger & Bard (1979; 1980) first demonstrated the surface transformation of a TTF.TCNQ electrode which produced symmetrical cathodic and anodic peaks because of reduction and oxidation of TCNQ⁰ and TTF⁰ respectively when cycled beyond -0.2 and +0.4 V. Therefore it is important not to allow the potential of the conducting salt to stray outside the stable region otherwise the electrode surface may become completely covered in insoluble decomposition products.

2.3.5 Coupling of methanol dehydrogenase with TTF.TCNQ

Prior to the investigation of the coupling of MDH with TTF.TCNQ, the stability of the organic conducting salt was confirmed by the observation of a featureless voltammogram obtained during the initial scan in the absence of MDH enzyme, substrate and activator (Scan (A), Figure 2.9).

Addition of 2 mg MDH, (from *Methylophilus methylotrophus*) in the absence of methanol substrate produced an anodic peak current of 0.17 μA at a potential of

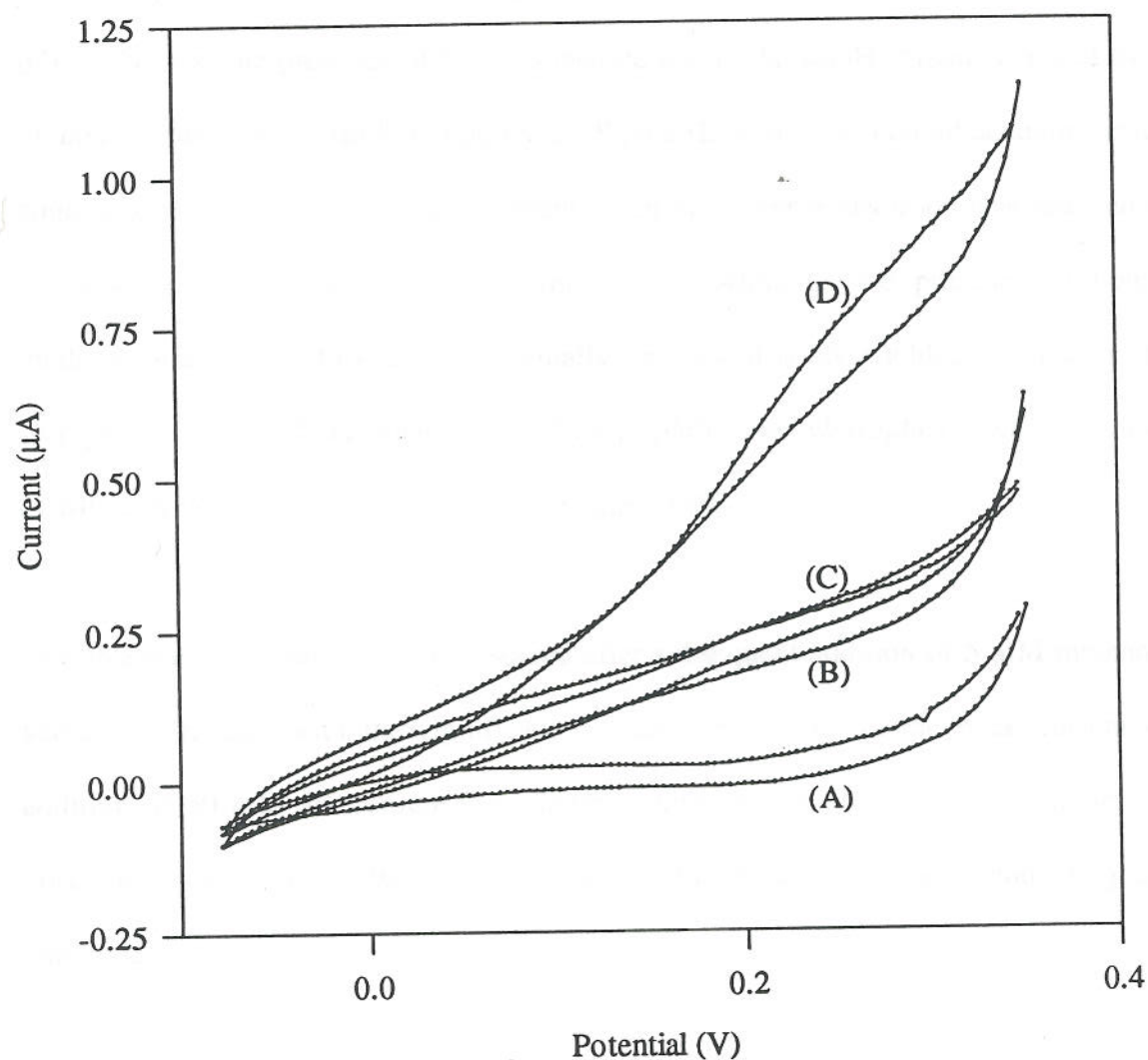


Figure 2.9. Cyclic voltammograms of MDH-TTF.TCNQ packed cavity electrodes.

Enzyme loading 2 mg MDH; Temperature = 30°C ; 0.2 M phosphate buffer, pH 8.0 ; 0.1 M KCl ; Potentials vs SCE reference electrode ; $v = 5 \text{ mV.s}^{-1}$

TTF.TCNQ electrode in buffer (A) ; TTF.TCNQ electrode plus enzyme (B) ; TTF.TCNQ electrode plus enzyme and 5 mM methanol (C) ; TTF.TCNQ electrode plus enzyme and 5 mM methanol in the presence of 50 mM ammonium ions (D).

MDH isolated from *Methylophilus methylotrophus*

+200 mV vs. S.C.E. (Scan (B), Figure 2.9). This was almost identical to the voltammogram obtained after subsequent addition of 5 mM methanol substrate (scan (C)). The possible presence of bound substrate within the MDH crystals isolated from *Methylophilus methylotrophus* supplied by Sigma (Dorset, U.K.) could account for the slight perturbation in anodic peak current when the enzyme electrode was scanned in the absence of methanol and ammonium ions. Although the presence of bound methanol was not confirmed experimentally, methanol is often added to preserve the storage stability of MDH isolated from *Methylophilus methylotrophus* since this source of MDH is very labile (Aston, 1984; Gossain, 1989).

The absence of current increase observed after subsequent addition of 5 mM methanol substrate, together with the significant 0.5 μ A increase in anodic peak current on addition of 50 mM ammonium ions, indicate that ammonium ions are necessary for efficient re-oxidation of the substrate reduced MDH at the organic conducting salt electrode.

Control experiments involving the independent addition of methanol and ammonium ions to the TTF.TCNQ electrode in the absence of MDH (*Methylophilus methylotrophus*) established that the organic conducting salt electrode does not oxidise the methanol substrate or ammonium ion activator directly.

Use of MDH isolated from *Paracoccus denitrificans* to investigate the coupling of MDH with TTF.TCNQ produced identical voltammograms to those observed in Figure

2.9 (A)-(D) for MDH isolated from *Methylophilus methylotrophus* (data not shown). As before, control experiments involving the independent addition of methanol and ammonium ions to the TTF.TCNQ electrode in the absence of MDH (*Paracoccus denitrificans*) established that the organic conducting salt electrode does not oxidise the methanol substrate or ammonium ion activator directly.

2.3.6 Stability of MDH-TTF.TCNQ packed cavity electrodes

Figure 2.10 illustrates the current response obtained after several repeat assays of MDH-TTF.TCNQ packed cavity electrodes using enzyme isolated from (a) *Methylophilus methylotrophus* (n=4) and (b) *Paracoccus denitrificans* (n=8). The change in current response observed during consecutive repeat assays provides an indication of the operational stability of each MDH-TTF.TCNQ packed cavity electrode.

As shown in Figure 2.10(a), the current response observed after four repeat assays of the same packed cavity electrode, prepared with MDH isolated from *Methylophilus methylotrophus*, decreased by more than 50% (maximum current: 0.591 μA 1st assay; 0.225 μA 4th assay). This significant decrease in current output observed during repeat assay of these electrodes can be attributed to the labile nature of MDH isolated from *Methylophilus methylotrophus*.

In contrast, very little change was observed in current response during eight consecutive assays of the same packed cavity electrode prepared with MDH isolated from *Paracoccus denitrificans* (maximum current: 0.531 μA 1st assay; 0.506 μA 8th assay;

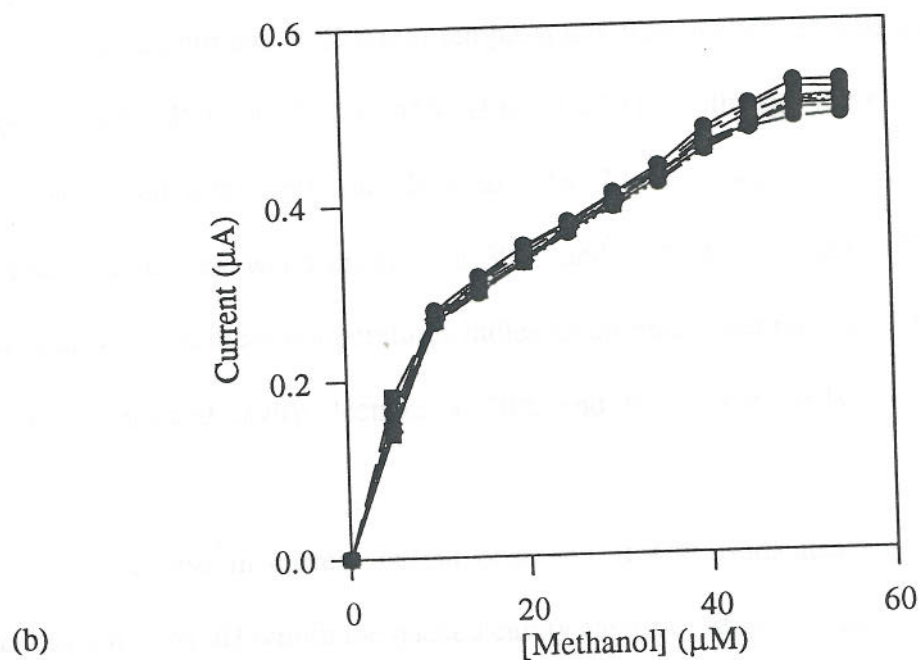
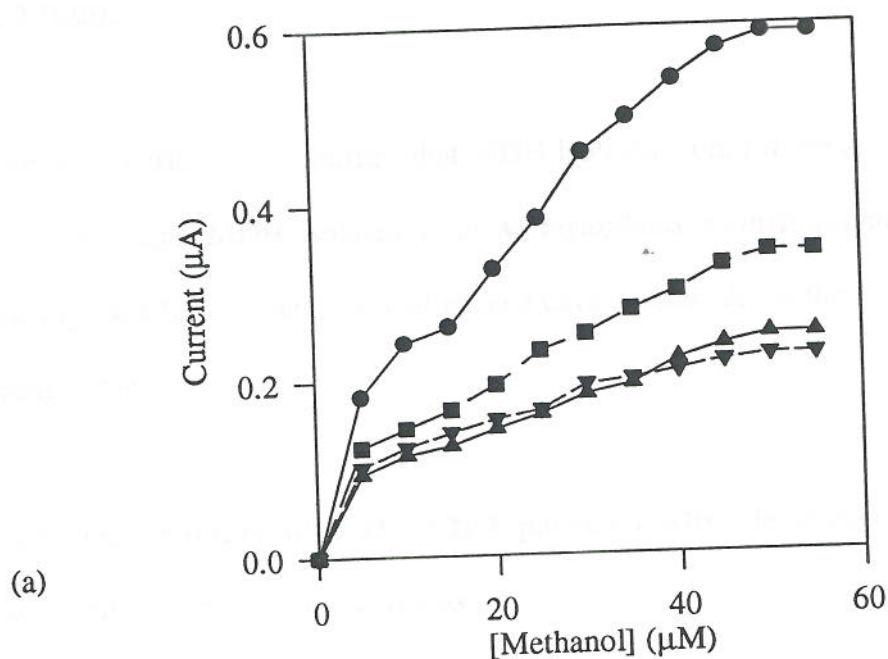


Figure 2.10. Operational stability of MDH-TTF.TCNQ packed cavity electrodes at 30°C. Enzyme isolated from (a) *Methylophilus methylotrophus* ($n = 4$), (b) *Paracoccus denitrificans* ($n = 8$). Electrodes assayed at +200 mV vs SCE in 0.2 M phosphate buffer, pH 8.0, 0.1 M KCl, in the presence of 50 mM ammonium ions.

1st assay —●— 2nd assay —■— 3rd assay —▲— 4th assay —▼—
 5th assay —●— 6th assay —◆— 7th assay —●— 8th assay —■—

Figure 2.10(b).

These amperometric results confirm that MDH isolated from *Paracoccus denitrificans* is more stable than MDH isolated from *Methylophilus methylotrophus* and can be successfully used for repeated assay of packed cavity electrodes without significant loss in current output.

2.3.7 Thermal stability of TTF.TCNQ packed cavity electrodes with enzyme isolated from *Paracoccus denitrificans*

2.3.7.1. Effect of temperature on current response

The order of increasing current response observed during assay of MDH-TTF.TCNQ packed cavity electrodes at different temperatures was unchanged 150 min after initial assays at 30°C, 35°C, 40°C, and 45°C (Figure 2.11). Although the maximum current output observed after 150 min decreased by 20 % at 40°C, the current response remained unchanged when assayed at 30°C and 35°C respectively. The absence of current decrease at these temperatures indicates optimum thermal stability of the MDH-TTF.TCNQ packed cavity electrode at 30°C and 35°C respectively.

The 57% decrease in current output observed at 45°C indicates significant thermal denaturation of MDH within the packed cavity electrode after 150 min incubation at this temperature. Complete thermal denaturation of MDH was observed after 90 min incubation at 50°C.

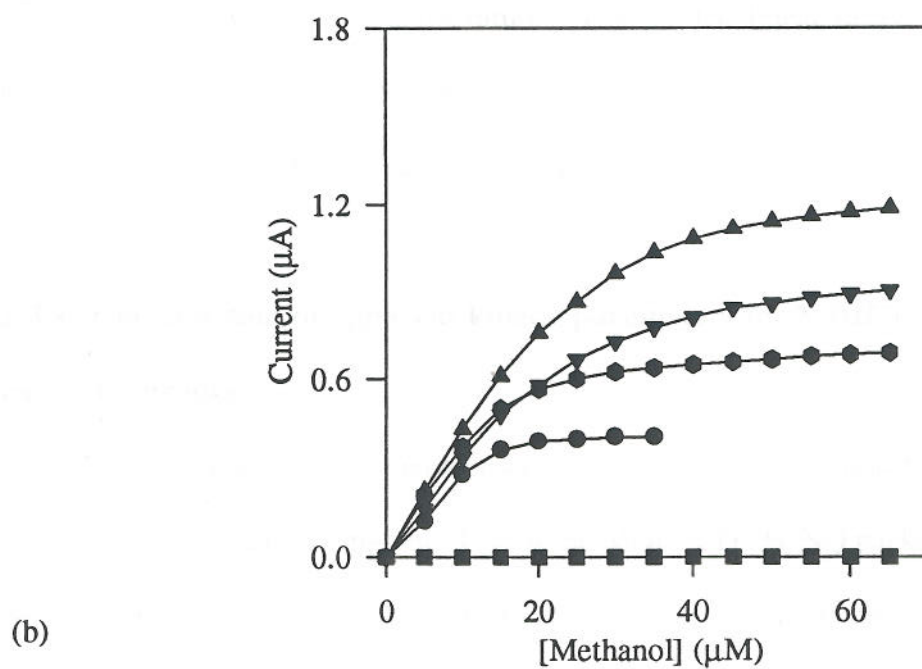
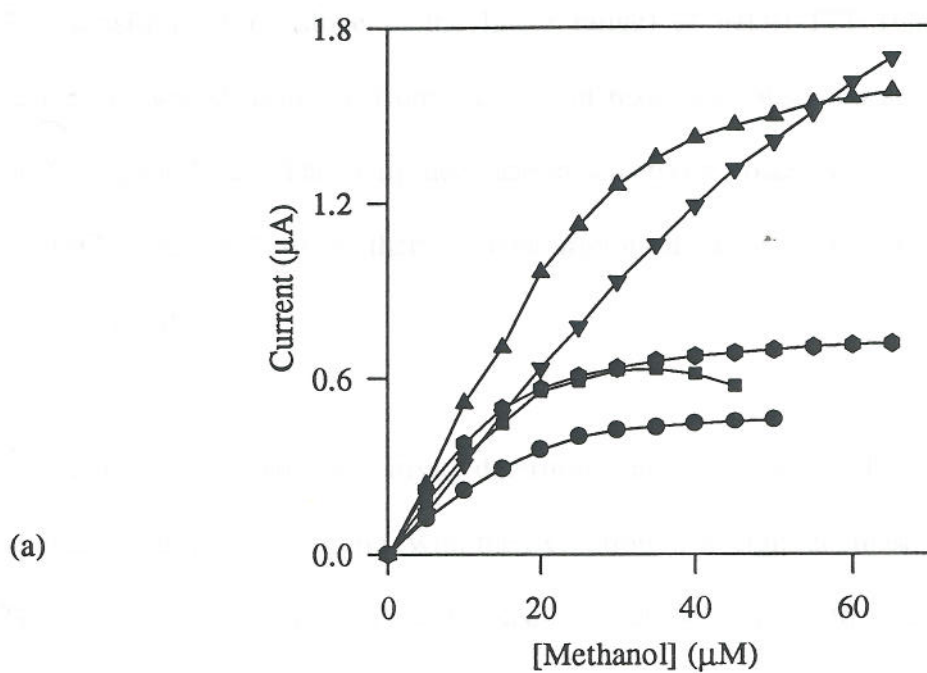


Figure 2.11. Current response at MDH-TTF.TCNQ paked cavity electrodes at (a) time zero and (b) after 150 min. Enzyme isolated from *Paracoccus denitrificans*. Electrodes assayed at +200 mV vs SCE in 0.2 M phosphate buffer, pH 8.0, 0.1 M KCl, in the presence of 50 mM ammonium ions, ● 30°C; ◐ 35°C; ▲ 40°C; ▼ 45°C; ■ 50°C.

2.3.7.2 Effect of temperature on sensitivity

The sensitivity (i.e., slope in the linear range) of MDH-TTF.TCNQ packed cavity electrodes was determined from the current responses observed at 30, 35, 40, 45 and 50°C (Figure 2.12). The sharp decrease in sensitivity observed after 90 min incubation at 50°C corresponds to the thermal denaturation of MDH after prolonged incubation at this temperature.

In general, the electrode sensitivity determined at 30, 35, 40 and 45°C remains relatively constant with respect to time (with the exception of a slight decrease in sensitivity after 60 min at 40°C). The optimal sensitivity at 40°C ($0.0346 \mu\text{A} \cdot \mu\text{M}^{-1}$) reflects the increased linear range (between 0 and 40 μM methanol) and higher current output (up to 1.7 μA) observed at this temperature. The limited linear range observed at lower temperatures ($\leq 20 \mu\text{M}$) accounts for the decreased sensitivity observed at 30°C and 35°C (0.024 and $0.028 \mu\text{A} \cdot \mu\text{M}^{-1}$) respectively.

2.3.8 Determination of apparent kinetic parameters for MDH-TTF.TCNQ packed cavity electrodes

2.3.8.1 Apparent kinetic parameters with respect to substrate addition

From Figure 2.13(a), the apparent K_M for the MDH-TTF.TCNQ packed cavity electrode was determined to be 0.02 mM (*Paracoccus denitrificans*) and 0.025 mM (*Methylophilus methylotrophus*). Both values are significantly less than the apparent K_M of 1 mM reported by Zhao and Lennox (1991) for a MDH (*Methylophilus methylotrophus*) packed cavity electrode in the absence of ammonium ions. The 50-fold

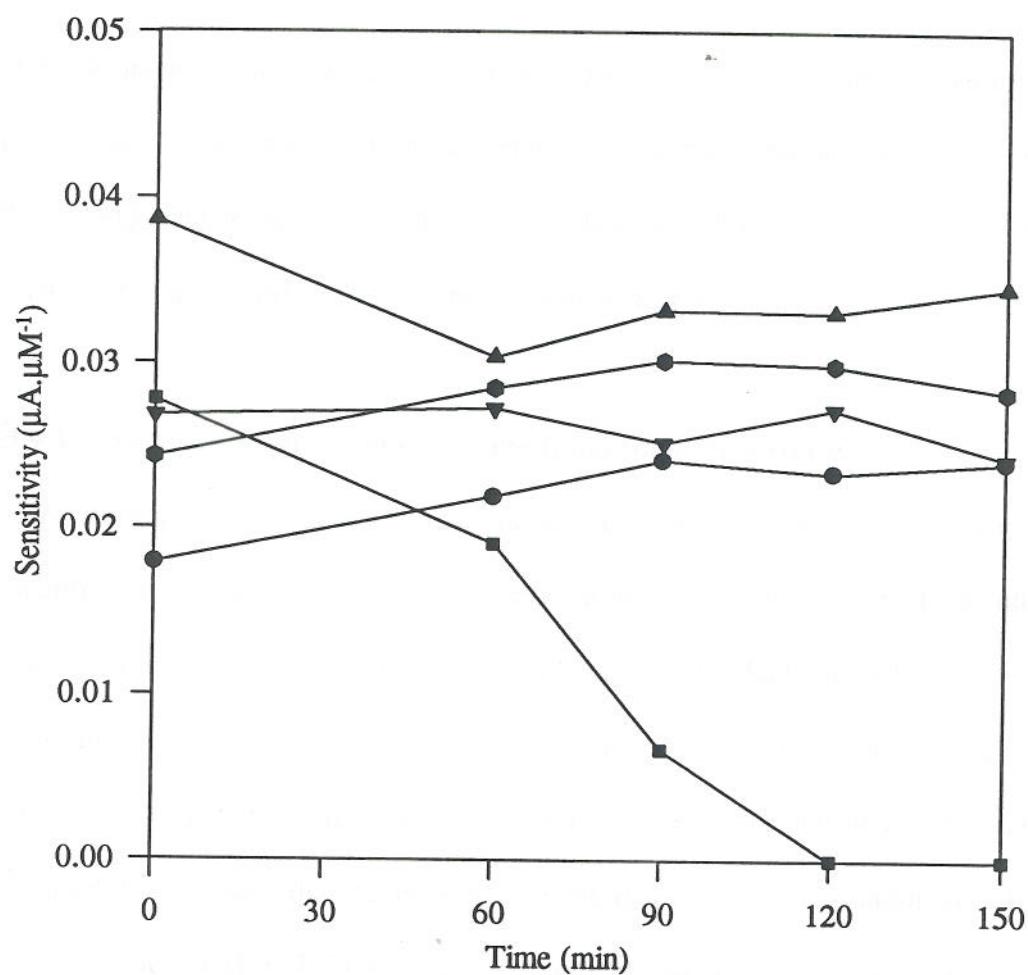


Figure 2.12. Effect of temperature on the sensitivity of MDH-TTF.TCNQ packed cavity electrodes. Enzyme isolated from *Paracoccus denitrificans*. Electrodes assayed at +200 mV vs SCE in 0.2 M phosphate buffer, pH 8.0, 0.1 M KCl, in the presence of 50 mM ammonium ions, ● 30°C; ● 35°C; ▲ 40°C; ▼ 45°C; ■ 50°C.

reduction of apparent K_M suggests an increased affinity of the enzyme electrode for methanol substrate in the presence of ammonium ion activator. This indicates that the binding of ammonium ion activator stimulates catalysis of methanol substrate.

2.3.8.2 Apparent kinetic parameters with respect to ammonium ion activation

From Figure 2.13(b) the apparent K_A (ammonium ion activation) values for the MDH-TTF.TCNQ packed cavity electrode were determined to be 0.05 mM (*Paracoccus denitrificans*) and 1 mM (*Methylophilus methylotrophus*).

2.3.9 Interactions of ammonia with methanol dehydrogenase

Determination of apparent kinetic parameters with respect to ammonium ion concentration, together with the increased catalytic currents observed on addition of ammonium ions to the MDH-TTF.TCNQ packed cavity electrode (Figure 2.9) indicate that ammonium ions are necessary for efficient re-oxidation of the substrate reduced MDH at the organic conducting salt electrode. This ammonium ion activation was supported by the fact that successive current responses to methanol could only be detected at the MDH-TTF.TCNQ packed cavity electrode in the presence of ammonium ions. Whilst small currents could be detected in response to a single aliquot of methanol in the absence of ammonium ions, subsequent addition of methanol produced no further increase in current output. However, in the presence of ammonium ions successive aliquots of methanol could be assayed at the MDH packed cavity electrode. Furthermore, control experiments in the absence of enzyme established that ammonium ions were not oxidized directly at the surface of the TTF.TCNQ organic conducting salt.

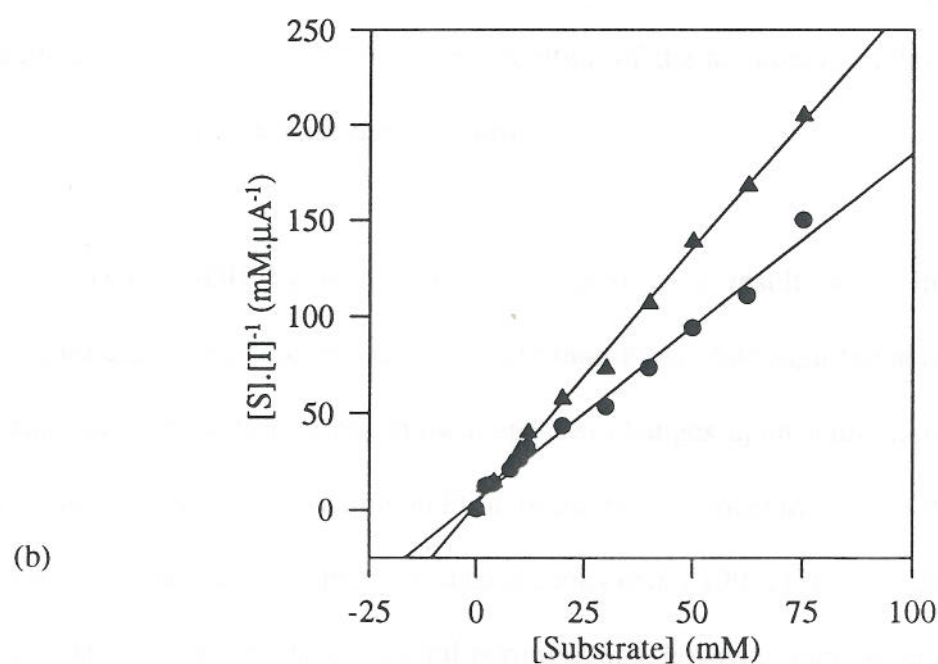
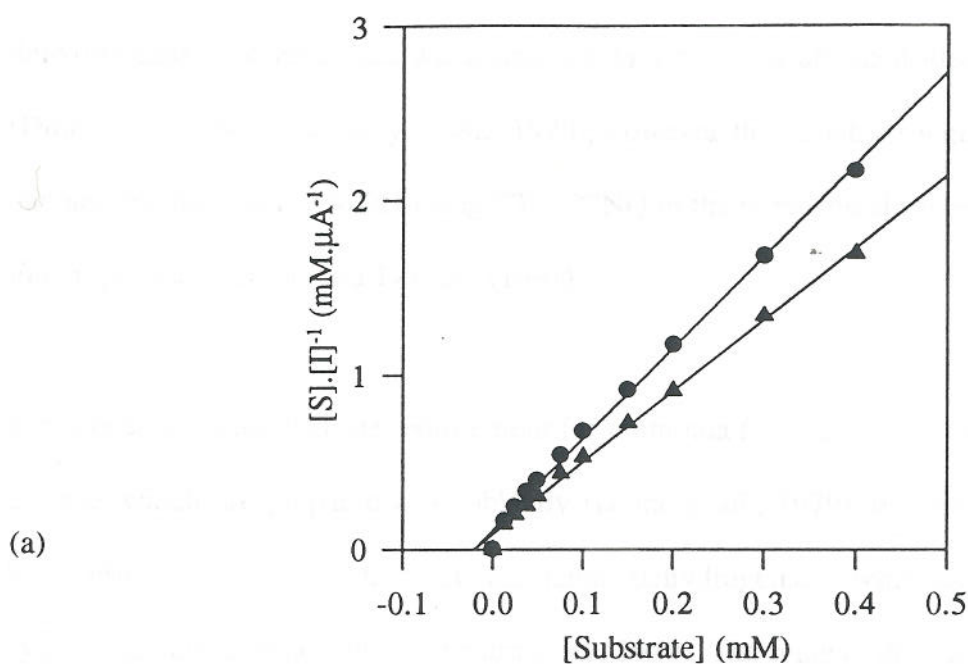


Figure 2.13. Hanes plot ($[S] / [I]$ vs $[S]$) for determination of apparent kinetic parameters of MDH-TTF.TCNQ packed cavity electrodes with respect to (a) methanol and (b) ammonium ions. Electrodes assayed in 0.2 M phosphate buffer, pH 8.0. 0.1 M KCl, at 30°C.

● *Methylophilus methylotrophus* MDH ; ▲ *Paracoccus denitrificans* MDH.

These observations are in good agreement with the absolute requirement of methanol dehydrogenase for ammonia when assayed *in vitro* with artificial electron acceptors (Duine *et al.*, 1979; Anthony, 1982; 1990), however they contrast with the anaerobic and aerobic turnover of MDH using TTF.TCNQ in the complete absence of ammonium ions reported by Zhao and Lennox (1991).

It has been reported that the requirement for ammonia for catalysis is reduced in crude extracts which are prepared anaerobically (Duine *et al.*, 1979) and that ammonia has little effect on the reaction of methanol dehydrogenase with its physiological cytochrome electron acceptor (Dijkstra *et al.*, 1989). The relative decrease in activation by ammonia observed when cytochrome was used as an electron acceptor reported by Dijkstra *et al.* (1989) may be due to the change in the rate-limiting step of the overall reaction which was assayed or to an alteration of the ammonia binding site when the enzyme is in complex with the cytochrome.

Activation of MDH by ammonia clearly must be a result of its interaction with methanol dehydrogenase at a different site than PQQ. Although the actual mechanism of ammonia interaction is not known, spectral changes upon iminoquinone formation are similar to those observed on addition of ammonia concentrations in the range of the K_i value obtained during kinetic studies (Brooks *et al.*, 1993; Harris & Davidson 1993). Other explanations for these spectral perturbations, however, cannot be ruled out. For example, ammonium ions may also act as counterions to oxyanions in the active site and in doing so perturb the micro-environment of the cofactor in such a way as to alter

the spectrum (Brooks *et al.*, 1993; Harris & Davidson *et al.*, 1993).

3. DETECTION OF HISTAMINE AT A TCNQ-METHYLAMINE DEHYDROGENASE ENZYME ELECTRODE

3.1 INTRODUCTION

Methylamine dehydrogenase (MADH) is a soluble bacterial enzyme which catalyses the oxidation of methylamine to formaldehyde and ammonia (Davidson, 1993).



As indicated in chapter one, (Section 1.3.4), MADH has a broad substrate specificity oxidising a wide range of primary amines to their corresponding aldehydes, and possesses tryptophan tryptophan quinone TTQ (Figure 1.1.1) as a covalently-bound prosthetic group (McIntire *et al.*, 1991; Chen *et al.*, 1991; 1992). This prosthetic group is derived from a post-translational modification of two gene encoded tryptophan residues (Chistoserdov *et al.*, 1990).

The physiological electron acceptor for MADH is a type I copper protein, amicyanin (Husain & Davidson, 1985). *In vitro*, the enzyme is typically assayed using either PES or PMS as an electron acceptor. These investigations involve the use of MADH isolated from *P. denitrificans* which exhibits optimal enzyme activity at pH 7.5, with K_M values of 10 μM for methylamine and 300 μM for PES. The V_{max} is 15.3 $\mu\text{mol min}^{-1}$ per mg of protein which corresponds to a k_{cat} of 32 s^{-1} (Husain & Davidson, 1987; Davidson, 1989).

Whilst detection of methylamine has limited commercial application, analysis of the primary amine histamine is an important tool when studying allergic responses and elevated histamine levels have been associated with a variety of pathological conditions including: gastric disorders, mastocytosis and chronic myelogenous leukaemia (Beaven *et al.*, 1982). Until recently, a biological assay was the only method available for determination of histamine in biological fluids. However a number of alternative methods have now been developed including HPLC analysis with a range of detection methods (Houdi *et al.*, 1987; Keyzer *et al.*, 1983); GLC (Wollin *et al.*, 1983) and a histamine sensitive membrane electrode (Katsu *et al.*, 1986).

Keyzer *et al.* (1983) used a nitrogen-phosphorous detector for GC determination of histamine metabolites in urine. This method enabled accurate determination of N^ε-methylhistamine in urine for clinical-chemical analysis without the requirement for sophisticated gas chromatographic-mass spectrophotometric equipment. Although the extraction and clean-up procedures were more laborious than previously described chromatographic techniques, this nitrogen-phosphorous GC determination of histamine metabolites would enable a single technician to analyse up to 40 urine samples within one day using an auto sampler (Keyzer *et al.*, 1983).

Measurement of histamine and some of its methylated metabolites in biological materials by gas liquid chromatography (GLC) at levels as low as 1.5 nmol/100 μ l was described by Wollin and Navert in 1985. The method was also adaptable to a variety of applications, with good reproducibility and sensitivity. However this method was

hindered by the lengthy sample pretreatment required prior to analysis. Four labour intensive and time-consuming preparation steps were required prior to GLC of histamine. These included initial ion-exchange chromatography to separate basic, neutral and acidic metabolites and to concentrate large sample volumes. Derivatisation with heptafluorobutyric acid followed by centrifugation were then required to ensure adequate volatility for GLC and sensitivity of the biological samples to both a nitrogen-phosphorous detector and electron capture detector. The nitrogen detector provides additional selectivity whereas electron capture detectors enhance the sensitivity but at the expense of selectivity (Wollin & Navert, 1985).

Whilst HPLC coupled with UV detection at 208-210 nm affords a separation of histamine and its metabolites this method of analysis is greatly limited by sensitivity. The preferred method for assaying histamine in biological fluids has been by fluorometric detection of its orthophthaldehyde (OPA) derivative. Since OPA reacts generally with primary amines, this method of analysis suffers from limited selectivity thus increasing the requirement for sample pretreatment and/or chromatographic resolution. Consequently Houdi *et al.* (1987) described a simple and sensitive method for the determination of histamine and its major metabolite N^ε-methylhistamine in biological fluids using HPLC coupled with electrochemical detection. Chromatographic separation of histamine and N^ε-methylhistamine was achieved within 20 min by ion-pair reversed-phase HPLC using a phosphate buffer:methanol mobile phase. Calibration curves showed linearity over 22-56 ng/ml for histamine and 250 ng/ml-5 µg/ml for N^ε-methylhistamine. About 15-20 analyses could be performed daily using this method;

during these analyses, no interferences were observed from the biological samples, indicating that the overall selectivity of this analytical method was good (Houdi *et al.*, 1987).

Katsu *et al.* (1986) constructed a histamine-sensitive membrane electrode using sodium tetrakis (p-fluorophenyl)borate as an ion-exchanger in conjunction with a σ -nitrophenylether membrane solvent for the detection of histamine release from mast cells. This electrochemical analysis using a selective ion-sensitive electrode enabled measurement of the histamine secretion process *in situ* without separating the histamine from the assay medium. Although this analysis has the inherent advantage of being simple and easy, the sensor membrane must be conditioned overnight in a solution containing 1 mM histamine, 10 mM NaCl, and 10 mM HEPES-NaOH buffer, pH 7.4. Although this ion selective electrode responded to histamine concentrations as low as 10 μ M it should be emphasized that the electrode was susceptible to large interference from other organic amines (Katsu *et al.*, 1986).

This chapter describes the preparation of amperometric enzyme electrodes for detection of methylamine and histamine. The equations for the biocatalytic and mediating reactions involved in histamine detection at a TCNQ-mediated quinoprotein dehydrogenase enzyme electrodes are shown in Figure 3.1. The mediator TCNQ facilitates electron transfer from MADH to a pyrolytic graphite carbon foil electrode. These methylamine dehydrogenase enzyme electrodes will potentially provide simple, rapid analysis of histamine without the need for extensive sample pretreatments

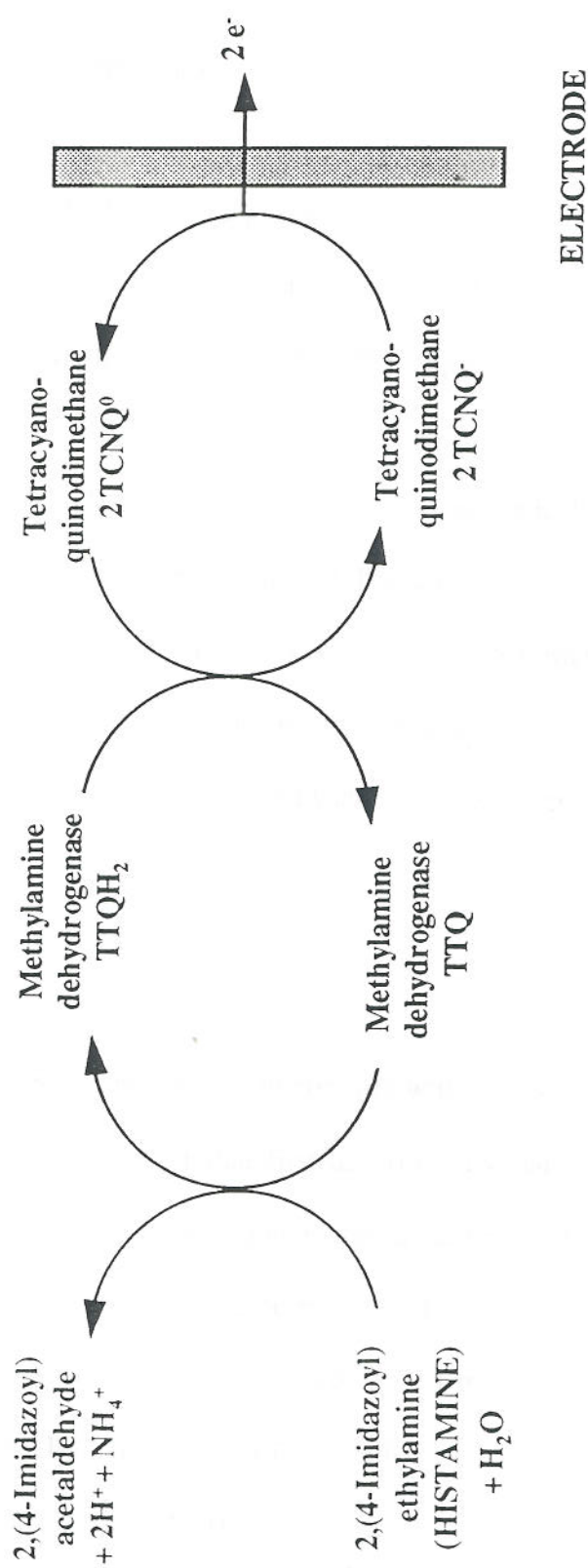


Figure 3.1. The biocatalytic and mediating reactions involved in histamine detection at a TCNQ-mediated methylamine dehydrogenase enzyme electrode.

currently required in HPLC and GLC analysis.

3.2. MATERIALS AND METHODS

3.2.1 Reagents

Histamine, methylamine hydrochloride, tetracyanoquinodimethane (TCNQ), phenazine ethosulphate (PES) and 2,6-dichlorophenol indophenol (DCPIP) were purchased from Aldrich Chemical Co., (Gillingham, Dorset, U.K.). Spectra/PorTM dialysis membrane, molecular weight cut off 15 000, was supplied wet in sodium azide by Pierce and Warriner (Chester, U.K.). The dialysis membrane was boiled in 1 % sodium carbonate, rinsed in deionised water and stored in 10 mM Tris containing 1 mM EDTA when not in use. Phosphate buffer (0.1 M) pH 7.5 was prepared in accordance with the method of Gomori *et al.* (1955). All chemicals were of Analytical Reagent grade and were used without further purification. Deionized water was used to prepare all standard and working solutions.

3.2.2 Solution kinetics

MADH activity was assayed spectrophotometrically with a dye-linked assay (Davidson 1990) which was a modification of that first described by Eady and Large (Eady & Large, 1968). Oxidised PES was used as an electron acceptor. It was then re-oxidised by DCPIP the reduction of which could be monitored by a decrease in absorbance at 600 nm. The standard assay mixture contained: 0.1 M potassium phosphate, pH 7.5; 4.8 mM PES; 0.17 mM DCPIP; either 0.1 M methylamine hydrochloride or 10 mM histamine substrate and 16 nM MADH.

The reaction was initiated by the addition of substrate and assays were performed at 30°C. Initial velocities were determined from the rate of reduction of DCPIP by measuring the change in absorbance at 600 nm.

3.2.3 Construction of enzyme electrodes

The base carbon electrodes were constructed as described previously (Cass *et al.*, 1984; Turner, 1988). Graphite foil discs 6 mm in diameter and 1mm thick (Le Carbonne, Portslade, Sussex) were fixed to 5.0 cm lengths of glass tube 6 mm in diameter using epoxy resin. The discs were washed twice in acetone (30 minutes for each wash with gentle agitation), once in boiling distilled water (1 hour) and dried in an oven at 100°C.

Electrical contact to the discs was made by cementing the disc to the exposed end of 0.2 mm diameter insulated wire of length 8.0 cm using silver loaded epoxy adhesive (RS Components Limited, Corby, Northants). Insulation of the electrical contact was made by covering the contact with about 100 µl of a mixture of 9 parts epon resin (grade 815) and 1 part triethylene tetramine catalyst (Polysciences, Inc., Northampton, Northants), and allowing it to set at 60°C over night.

Electrodes were polished with an alumina water slurry (particle size 0.2 µm, BDH, Merck Limited, Poole, Dorset) then cleaned electrochemically in 1 M H₂SO₄, by cycling between -0.8 V and +1.4 V in accordance with the method of Sawyer and Roberts (1974). Electrodes were thoroughly rinsed in distilled water prior to adsorption of 4.9 µmoles TCNQ dissolved in acetonitrile. The acetonitrile solvent was allowed to

evaporate (30 min.) before addition of 2.92 nmoles of MADH (in 40 μ l). The enzyme electrode was then covered with 1 cm² Spectra/PorTM dialysis membrane held in place with a 6 x 2 mm "o"-ring.

3.2.4 Electrode Measurement Apparatus

A three electrode system was used in conjunction with a precision potentiostat (Ministat, H.B. Thompson and Associates, Newcastle upon Tyne). The current was recorded on a Y/t chart recorder via a resistance board (JJ Instruments, Southampton, Hants). A saturated calomel electrode (S.C.E.) supplied by Russell (Auchtermuchty, Fife) was used as a reference and the auxiliary electrode was a 0.46 mm diameter platinum wire (BDH, Merck Limited, Poole, Dorset).

The enzyme electrodes were placed in 10 ml of 0.1 M phosphate buffer, pH 7.5, (containing 0.1 M KCl supporting electrolyte) contained in a 20 ml glass water-jacketed cell (Soham Scientific, Ely, Cambs.) thermostated at 30°C. The sensors were poised at +200 mV vs. S.C.E. for amperometric detection of current upon the addition of methylamine and histamine substrates.

3.2.5 Electrode characterisation

The MADH-TCNQ electrodes constructed as described in section 3.2.3 were characterised with respect to:

- i the sensitivity (slope of the calibration curve) and linear range of current response to methylamine and histamine substrates;
- ii limit of detection for histamine determined with respect to signal-to-noise ratio of 3:1 in accordance with standard definition of clinical chemical analysis (Buttner *et al.*, 1981);
- iii within batch precision determined for 10 measurements at 70 μM histamine;
- iv assessment of potential interferents: primary amines and other nitrogenous compounds prevalent in blood and plasma which might inhibit practical application of MADH for analysis of histamine in biological fluids;
- v determination of apparent kinetic parameters for methylamine and histamine substrates.

3.3 RESULTS AND DISCUSSION

3.3.1 Solution studies of MADH

Although the primary concern of this research was to investigate the possibility of using MADH for detection of histamine, initial studies with methylamine helped to characterise the activity of MADH in solution. In this respect comparison of the respective Michaelis constants with literature values provided an indication of the enzyme's affinity for each substrate.

Steady-state kinetic analysis of MADH using a spectrophotometric assay yielded a K_M of 12.5 μM for methylamine (Figure 3.2a) in good agreement with 10 μM previously observed by Husain and Davidson (1987). Similar steady-state kinetic analysis with respect to histamine provided a K_M of 1.83 mM (Figure 3.2b) which also compares favourably with the 1.5 mM determined by Davidson (1989). The observed difference in the Michaelis constants determined for each substrate (two orders of magnitude) reflects the fact that MADH has a much higher affinity for methylamine substrate. This is not unreasonable since methylamine is the primary substrate for MADH catalysis. However, since MADH reacts with histamine in solution and consistent kinetic parameters can be determined with respect to MADH histamine catalysis, this indicates the possible incorporation of MADH into an enzyme electrode for detection of histamine.

As part of this investigation the spectral change on addition of histamine dihydrochloride to methylamine dehydrogenase was studied by Victor Davidson

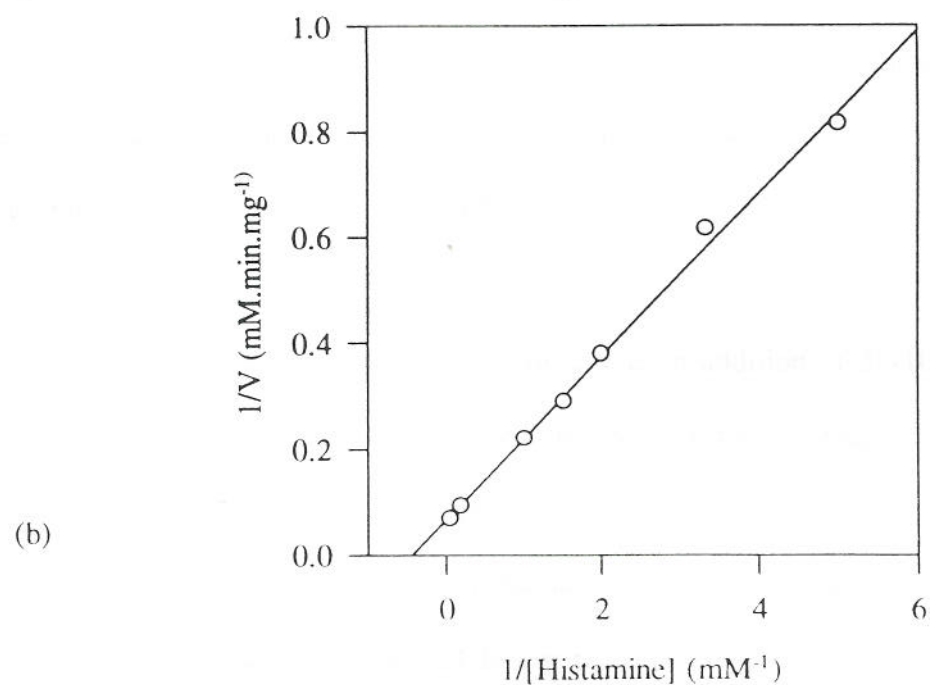
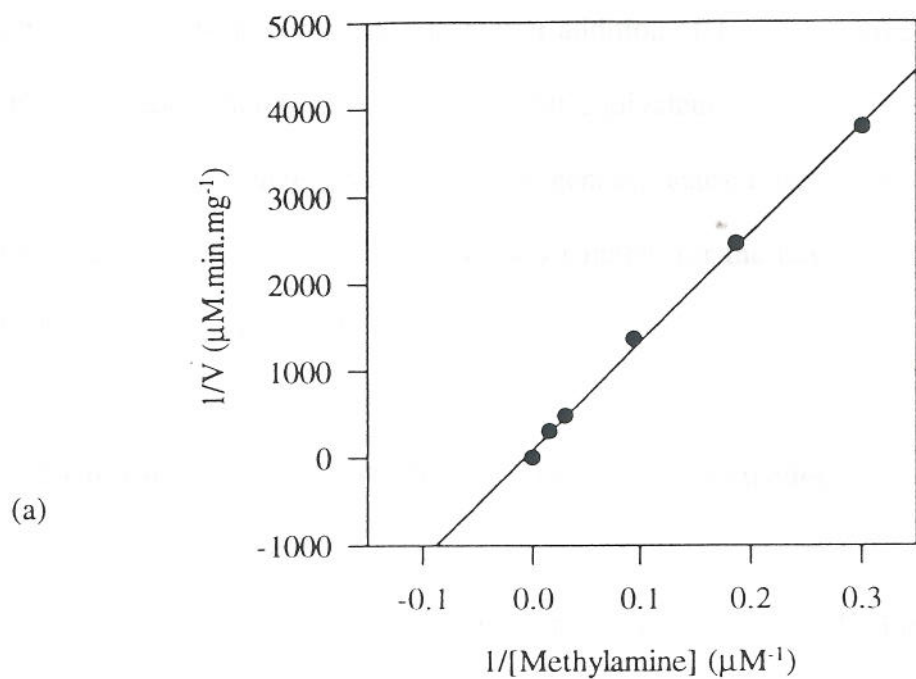


Figure 3.2. Lineweaver-Burk plot ($1/[S]$ vs $1/V$), for determination of kinetic parameters of methylamine dehydrogenase in solution with respect to (a) methylamine and (b) histamine substrates.

(Appendix One, personal communication). Spectra of oxidized methylamine dehydrogenase were recorded, 2 min after addition of 1 molar equivalent of histamine, and 8 min after addition of a second molar equivalent. The observed spectral change in redox state of methylamine dehydrogenase, caused by oxidation of histamine substrate, confirmed that the enzyme was suitable for the development of an enzyme electrode for detection of histamine.

3.3.2 Characterisation of MADH-TCNQ enzyme electrodes

3.3.2.1 Calibration of enzyme electrodes

As illustrated in Figure 3.3 the current response increased on addition of methylamine or histamine to TCNQ-modified methylamine dehydrogenase enzyme electrodes. The current response observed on addition of methylamine was almost twice the response observed for similar concentrations of histamine (upto 4.24 μA cf. 2.7 μA respectively). Although a non-linear response was observed at methylamine concentrations $< 100 \mu\text{M}$, the response was almost linear between 150 and 500 μM before reaching saturation at methylamine concentrations $< 550 \mu\text{M}$.

A non-linear current response was also observed on addition of 50-1000 μM aliquots of histamine to MADH-TCNQ modified enzyme electrodes. The non-linear response in Figure 3.3 between 0 and 200 μM substrate indicates inefficient electron transfer between reduced TCNQ and the electrode surface at low substrate concentrations. This inefficient electron transfer observed during initial investigations of the TCNQ-mediated electrode response to methylamine and histamine may be due to the presence of low

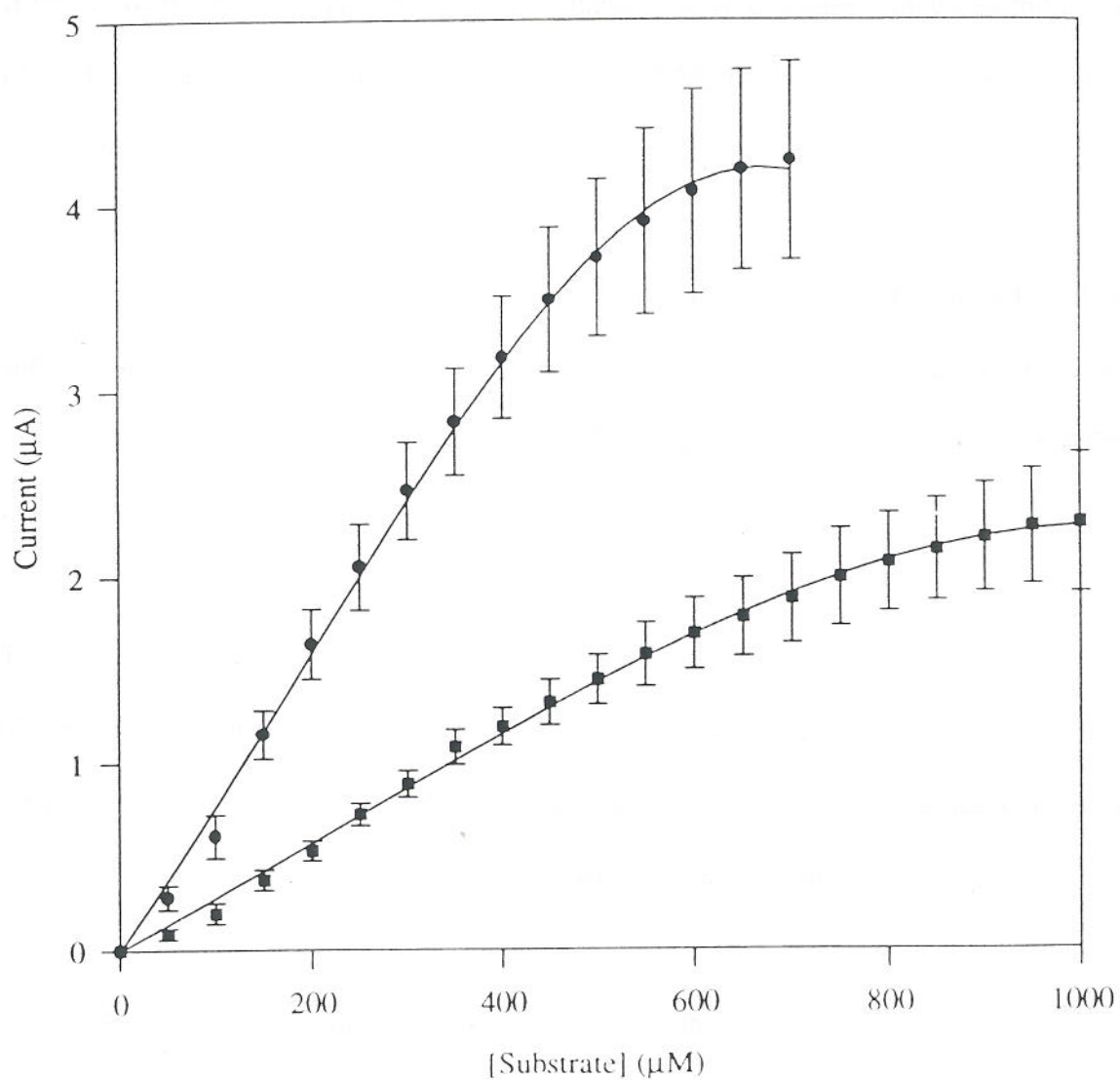


Figure 3.3. Calibration curves for detection of ● methylamine and ■ histamine at TCNQ modified methylamine dehydrogenase enzyme electrodes. Error bars represent standard deviation ($n = 5$).

molecular weight cytochrome contaminants (M_r 12 000) within the MADH preparation (Davidson, personal communication). Although this could not be confirmed by spectral analysis, the enzyme was passed through an AmiconTM membrane filter (Molecular weight cut off 10 000) supplied by AmiconTM Ltd. (Stonehose, Gloucestershire). Ultra-filtration of the MADH with the AmiconTM membrane filter then enabled determination of the linear range and sensitivity of the MADH-TCNQ modified electrode with respect to histamine (Figure 3.4).

It should be emphasized that the MADH electrodes investigated in Figure 3.4, and all subsequent analyses with respect to histamine, were all prepared with the same concentration of MADH used in section 3.2.3 (i.e., addition of 2.92 moles of MADH in 40 μ l).

The current response observed on addition of successive 10 μ M aliquots of histamine (Figure 3.4) provided a linear range between 0 and 140 μ M. The associated current output, between 0 and 0.865 μ A, enabled determination of the electrode sensitivity (slope in the linear range) as 6.18 nA. μ M⁻¹ with respect to histamine.

3.3.2.2 *The limit of detection with respect to histamine*

The limit of detection determined with respect to signal-to-noise ratio of 3:1 in accordance with the standard definition of clinical chemical analysis (Buttner *et al.*, 1980) was 4.8 μ M histamine. This value compares favourably with the lower limits of detection reported for a potentiometric histamine sensitive membrane electrode (10 μ M,

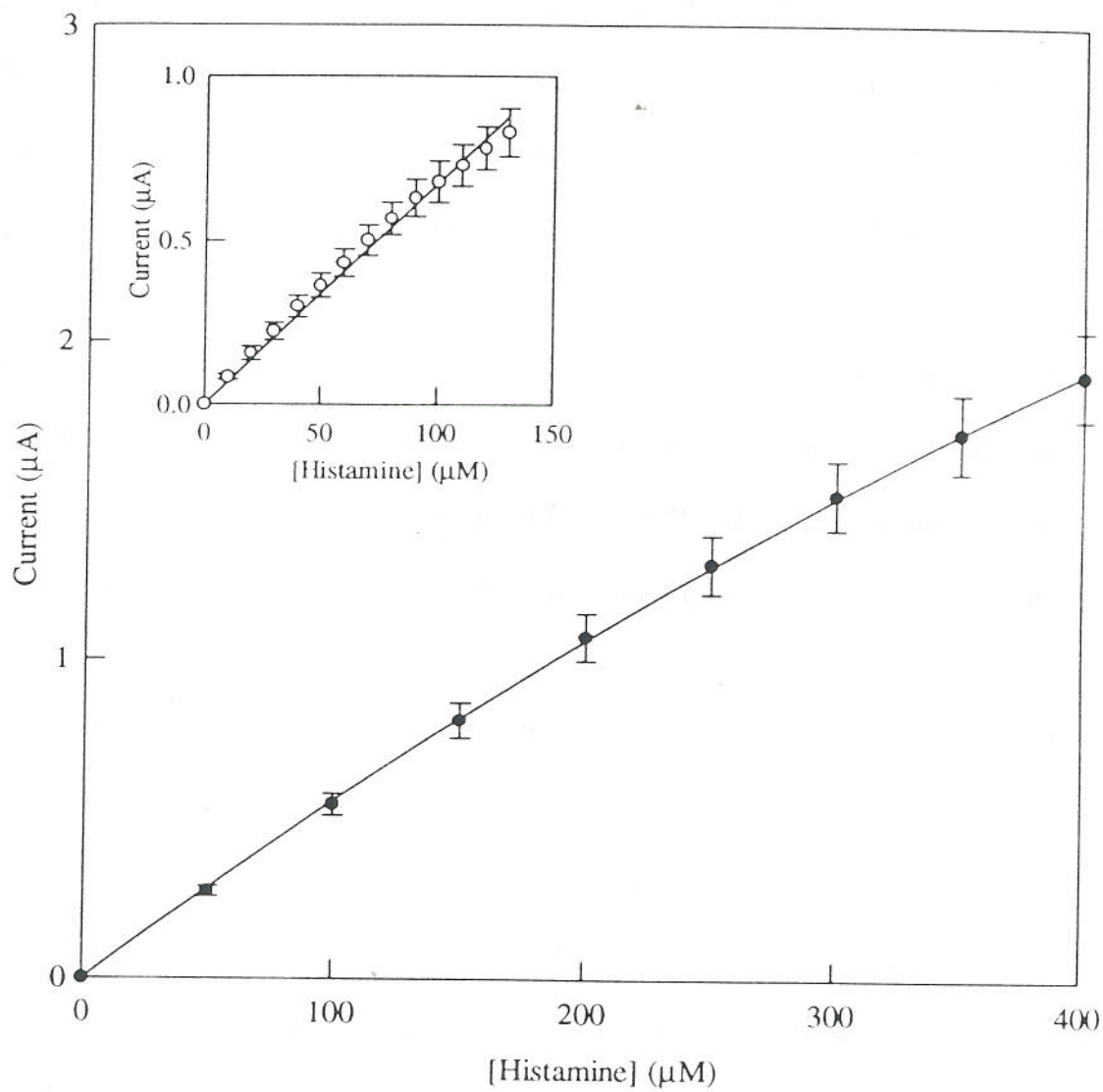


Figure 3.4. Linear range for histamine detection at TCNQ modified methylamine dehydrogenase enzyme electrodes. Inset, amperometric assay of successive $10\ \mu\text{M}$ aliquots of histamine.

Katsu *et al.*, 1986).

3.3.2.3 The precision of histamine measurement at MADH-TCNQ enzyme electrodes

The within batch precision of the MADH-TCNQ enzyme electrode current response was determined for 10 consecutive measurements at 70 μM histamine. The mean current output was $0.438 \mu\text{A} \pm 0.0151$, with a coefficient of variation of 3.45 %, $n = 10$ (Table 3.1). The selected histamine concentration of 70 μM corresponds to the lower range of histamine prevalent in patients with chronic myelogenous leukaemia ($< 243 \mu\text{M}$, Beaven *et al.*, 1982) and lies in the linear portion of the calibration curve at low histamine concentrations ($< 150 \mu\text{M}$, inset Figure 3.4). These results indicate that this MADH-TCNQ electrode could be applied for future clinical analysis of histamine.

3.3.3 Potential application of MADH-TCNQ enzyme electrodes for analysis of histamine in biological fluids

Beaven *et al.* (1982) listed histamine levels in plasma, blood and urine in control subjects and patients with pathological conditions related to histamine release. From their data normal blood histamine levels are $\geq 0.1 \mu\text{M}$, whilst those quoted for a range of pathological disorders include: mastocytosis $\geq 0.7 \mu\text{M}$; polycythemia vera $\geq 6 \mu\text{M}$; chronic myelogenous leukaemia $\geq 243 \mu\text{M}$ (Beaven *et al.*, 1982).

Methylamine dehydrogenase is not only sensitive to histamine but catalyses the oxidation of all primary amines including methylamine, ethylamine, propylamine and butylamine, to their corresponding aldehydes and ammonia (Husain & Davidson, 1987;

Electrode (No.)	Current (μA)
1	0.453
2	0.453
3	0.463
4	0.421
5	0.432
6	0.421
7	0.443
8	0.443
9	0.421
10	0.432
Mean $\pm$ S.D.	0.438 $\pm$ 0.0151
Coefficient of variation	3.45 %

Table 3.1: Within batch current responses of TCNQ-modified MADH electrodes to 70 μM histamine.

Davidson, 1989). No significant amounts of the primary amines quoted above are present in blood or plasma (Long, 1971), consequently an interference problem is not anticipated by either methylamine, ethylamine, propylamine or butylamine.

The lack of activity of MADH with the amino acids lysine and valine (Husain & Davidson, 1987) indicated that interference from other amino acids may also be unlikely. In this respect, no current response was observed when the TCNQ-MADH electrode was assayed with physiological concentrations of glutamine ($9.1 \text{ mg.100ml}^{-1}$); creatinine ($1.28 \text{ mg.100ml}^{-1}$) and creatine ($3.9 \text{ mg.100ml}^{-1}$) as shown in Table 3.2. Amperometric responses of the TCNQ-MADH electrode to physiological concentrations of urea and ascorbic acid, two compounds commonly associated with interference at enzyme electrodes, were also investigated (see Table 3.2).

Whilst urea did not pose an interference problem, the amperometric response to $70 \text{ }\mu\text{M}$ concentrations of ascorbic acid ($0.268 \text{ }\mu\text{A}$) was 60 % of the response observed for equivalent concentrations of histamine. Since ascorbic acid interference has also been observed at enzyme electrodes constructed for detection of glucose, lactate and fructose (Sasso *et al.*, 1990; White *et al.*, 1992; Xie *et al.*, 1991), the interference at the MADH-TCNQ electrode was not unexpected.

Regarding the specific application of this TCNQ-MADH enzyme electrode for detection of elevated histamine levels prevalent in chronic myelogenous leukaemia ($450 - 27,000 \text{ ng.ml}^{-1}$), all the potential nitrogenous interferents (primary amines and amino acids)

Substrate	Concentration mg.100ml ⁻¹	Response
Glutamine	9.1 (623 μ M)	-
Creatine	3.9 (297 μ M)	-
Creatinine	1.28 (113 μ M)	-
Urea	28.6 (4720 μ M)	-
Ascorbic acid	1.0 (57 μ M)	60 %
Histamine	7.35 (70 μ M)	100 %

Table 3.2: Amperometric assay of potential interferents at corresponding physiological concentrations (Long, 1971) for TCNQ-modified MADH electrodes (n = 3). The response at 70 μ M histamine, which corresponds to 100 %, was 0.447 μ A in good agreement with the precision data.

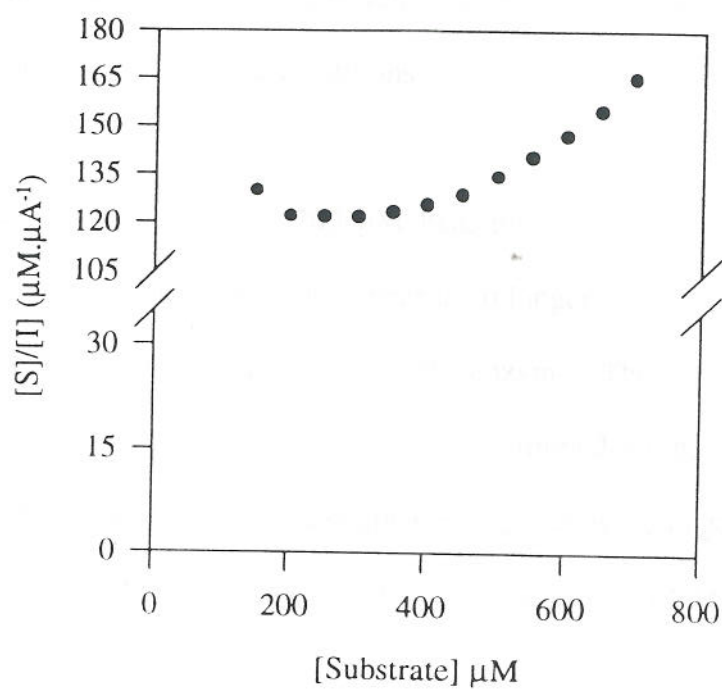
listed in Table 3.2 are confined to plasma with the exception of creatinine. Therefore interference may not be a problem for analysis of histamine in blood from which plasma has been removed during fractionation of specific blood components. However, some interference problems must still be overcome, in particular polyamine concentration is $> 20 \mu\text{M}$ in blood, $> 0.2 \mu\text{M}$ in plasma (Long, 1971), and it increases in pathological conditions. It remains to be seen whether polyamines such as spermine, spermidine or putrescine cause interference at the MADH-TCNQ electrode.

Further work is necessary to eliminate ascorbic acid interference at TCNQ-MADH electrodes in accordance with techniques which have been previously employed to reduce or eliminate ascorbic acid interference at different enzyme electrodes. These include : co-immobilisation with ascorbate oxidase (Xie *et al.*, 1991); use of platinised carbon and/or chemically modified electrode surfaces to enable enzyme electrodes to be operated at a lower applied potential where ascorbic acid interference is minimised, (White, *et al.*, 1994, Jaffari 1994); use of selectively permeable membranes such as Nafion[®] and cellulose acetate to exclude ascorbic acid from the electrode surface (Dong *et al.*, 1991; Lowry & O'Neill, 1992).

3.3.4 Determination of apparent kinetic parameters for TCNQ-MADH enzyme electrodes

A Hanes plot showed biphasic kinetics with respect to methylamine (Figure 3.5a) and histamine (Figure 3.5b). This reflects limiting diffusion of both substrates across the membrane at low substrate concentrations ($< 200 \mu\text{M}$ histamine, $< 400 \mu\text{M}$

(a)



(b)

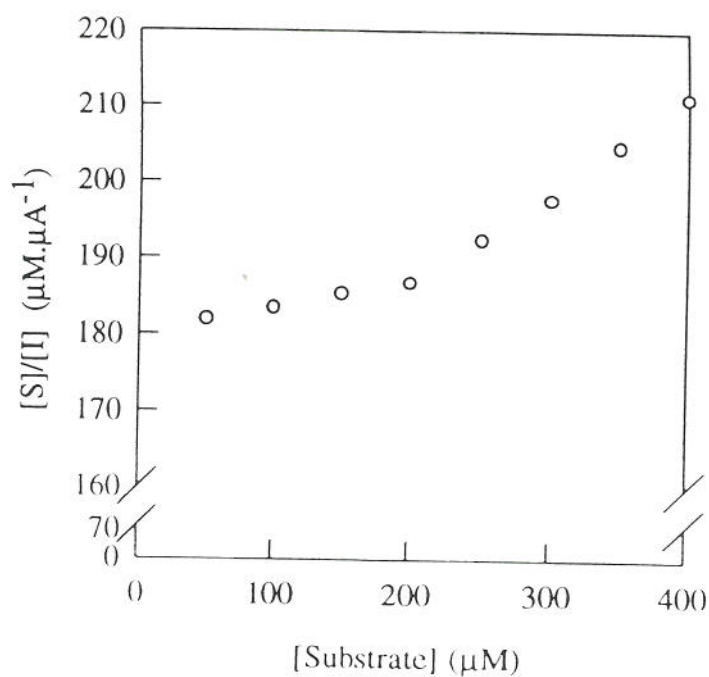


Figure 3.5. Hanes plot ($[S]/[I]$ vs $[S]$) for determination of apparent kinetic parameters of TCNQ-modified methylamine dehydrogenase enzyme electrodes with respect to (a) methylamine and (b) histamine substrates.

methylamine) confirmed by the high apparent K_M (5.6 mM histamine, 10.4 mM methylamine) determined under these conditions.

At higher substrate concentrations ($> 200 \mu\text{M}$ histamine, $> 400 \mu\text{M}$ methylamine) diffusion of substrate across the membrane barrier is no longer limiting and the current becomes principally dependent on the kinetics of the enzyme. The apparent K_M of the membrane electrode under these conditions was determined to be 1.35 mM for histamine and 25 μM for methylamine. These apparent K_M values are in good agreement with the K_M determined for MADH and both substrates in solution (1.83 mM histamine, 12.5 μM methylamine, section 3.3.1).

4. DEVELOPMENT OF PYRROLOQUINOLINE QUINONE MODIFIED ENZYME ELECTRODES

4.1 INTRODUCTION

4.1.1 Polypyrrole-pyrroloquinoline quinone electrochemistry

Shinohara *et al.* (1991) demonstrated that the reversible electrochemistry of the PQQ redox reaction could be obtained with the use of polypyrrole modified platinum electrodes. Using 3 mM PQQ at a scan rate of 2 mV.s⁻¹ a peak shaped voltammogram was shown with a peak separation of 35 mV. The corresponding anodic peak current was proportional to the square root of the scan rate in accordance with the Nicholson Shain theory of electron transfer (Nicholson & Shain, 1964) which indicated that the electrochemistry of PQQ was diffusion controlled. The diffusion coefficient was calculated to be $1.9 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, in accordance with the Randles-Sevcik equation. Consequently Shinohara *et al.* proposed PQQ-polypyrrole modified electrodes as a route for the manufacture of biosensors for glucose, fructose, galactose incorporating PQQ enzymes. The aim of the current work was to assess the suitability of polypyrrole membrane electrodes for development of PQQ-modified enzyme electrodes.

4.1.2 Coupling of pyrroloquinoline quinone with glucose oxidase

Glucose oxidase is a dimeric protein with a molecular weight of 160 kDa, containing one tightly bound flavin adenine dinucleotide (FAD) per monomer as cofactor. The FAD molecule is localized in a cavity deeper than 8 Å rather than being accessible to direct electrode contact on the molecule surface. This means that it is unlikely that

FAD, directly bound to the electrode forms the holoenzyme by reaction with the apoenzyme.

Under normal circumstances the transfer of electrons between the active site of reduced glucose oxidase and an electrode takes place slowly or not at all (Bogdanovskaya *et al.*, 1988, Szuks *et al.*, 1989). This is because the gap between the active site and the electrode is too large for the electrons to cross. Transfer of electrons between the active site and the electrode can be facilitated if a small electron acceptor is used as a mediator (Turner, 1988; Hill & Sanghera 1990).

In this chapter, the aim is to use PQQ as a mediator to facilitate the transfer of electrons from glucose oxidase to the electrode. The tertiary structure of glucose oxidase is characterised by two separate and distinctly different β -sheet systems, one of which forms part of the FAD binding domain. The second is a large six-stranded antiparallel β -sheet supported by four α -helices on its back. This β -sheet forms one side of the active site. The two isoalloxazine moieties are separated by a rather large distance of about 40 Å, a distance which excludes any electrical communication between them (Hecht *et al.*, 1993). From the tertiary structure, the minimum distance between the flavin and the surface of the monomer is more than 13 Å. This means that in the dimer the distance is even larger. This represents a significant distance for electrons to be transferred between the FAD active site of glucose oxidase and the unprotonated PQQ to initiate electron transfer complex formation and catalysis (Hecht *et al.*, 1993).

4.2 MATERIALS AND METHODS

4.2.1 Reagents

PQQ was purchased from Sigma (Poole, Dorset) and was used as supplied. Pyrrole was purchased from Aldrich Chemical Company (Poole, Dorset) and distilled before use as described in section 4.2.4. Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* (Glucosyl PS: activity 75,000 U/g) was purchased from Rhône-Poulenc Chemicals Ltd. (Stockport, U.K.). All other reagents (citrate, sodium phosphate and potassium chloride) were supplied by BDH (BDH, Merck Limited, Poole, Dorset) and were of analytical grade.

4.2.2 Determination of electrode area

The electrochemical area of platinum foil working electrodes was determined to enable control of the coverage with polypyrrole with respect to the accumulation of charge on the electrode surface (charge density, $C \cdot cm^{-2}$). The effective electrochemical area of the platinum working electrode was determined by performing chronocoulometry on a redox species of known diffusion coefficient (potassium hexacyanoferrate-(II), $K_4Fe(CN)_6$). The working electrode was immersed in a cell containing 1 mM potassium hexacyanoferrate-(II) in 1 M potassium chloride (5 ml). A potential of +450 mV vs SCE was maintained for 6.5 seconds and the increase in charge over this time period was recorded. Since the charge passed (Q) is directly proportional to $t^{1/2}$ (Anson, 1967), the electrochemical area can be determined from a plot of Q vs. $t^{1/2}$ in accordance with the integrated Cottrell equation (Section 4.3.2).

4.2.3 Preparation of pyrrole

Pyrrole (10 ml aliquots) was distilled under reflux before use. Distillation was carried out in a fume cupboard using round bottomed flasks attached to a distillation column and Leibig condenser. Clear yellow pyrrole droplets were collected from the original black cloudy solution after 45 min. Aliquots (0.1 M) of the fresh distilled pyrrole were then used for chronocoulometric polymerisation of pyrrole and entrapment of PQQ as described in section 4.2.4.

4.2.4 Chronocoulometric polymerisation of pyrrole and entrapment of PQQ

A 1 ml aliquot of PQQ (3 mM) was prepared in 0.1 M KCl pH 8.0, 50 μ L of 0.1 M KOH was added to dissolve the PQQ completely by adjusting the pH from 8 to 9. This PQQ solution was contained in a single compartment electrochemical cell together with an Ag/AgCl reference electrode, a Pt foil working electrode (Electrochemical area 0.313 cm²), and a 0.46 mm diameter Pt wire c.e. The electrochemical cell was purged with nitrogen for 30 min to remove oxygen and prevent auto-oxidation of the distilled pyrrole, and was thermostated at 25°C by a circulating water bath (Gallenkamp, Loughborough, Leics.). After 30 min the nitrogen line was maintained just above the surface of the electrochemical solution in order to maintain the deoxygenation and 8 μ L (0.1 M) of fresh distilled pyrrole was added.

Polymerisation of the pyrrole and entrapment of the resultant PQQ were carried out simultaneously (via chronocoulometry) by applying a potential of +800 mV vs. Ag/AgCl reference electrode until the desired amount of charge had passed (31.3 mC

$\equiv 100 \text{ mC.cm}^{-2}$).

4.2.5 Cyclic voltammetry of glucose oxidase and PQQ

Electrochemical measurements were performed using an Autolab Electrochemical Analyser (Windsor Scientific, Windsor) interfaced to a Viglen personal computer (Viglen Ltd., London) and an Epson LX-400 printer (Epson Ltd., Telford) as described in section 2.2.2. Before each voltammetric experiment the working electrode was cleaned by polishing with an alumina water paste (particle size $0.3 \mu\text{m}$, BDH Merck Ltd., Poole, Dorset) on cotton wool, followed by washing with de-ionized water (Cass *et al.*, 1984). All the measurements were carried out at 25°C in a two compartment electrochemical cell (Soham Scientific, Ely, Cambs.) shown in Figure 4.1.

The working compartment contained a platinum working electrode supplied by Biotech Instruments (Luton, U. K.) and a platinum gauze auxiliary electrode immersed in 3 mM PQQ containing 50 mM glucose, at the desired pH. The reference compartment was connected to the working compartment via a Luggin capillary and contained an Ag/AgCl reference electrode (Biotech Instruments, Luton, U.K.) immersed in 3 ml 0.1 M potassium chloride electrolyte. All potentials are reported with respect to the Ag/AgCl reference electrode unless otherwise stated. Solutions in the electrochemical cell were purged with nitrogen for 30 minutes to remove oxygen. Citrate buffer (0.1M with addition of 0.1 M KCl) was titrated with HCl or NaOH directly in the electrochemical cell to obtain the required pH values.

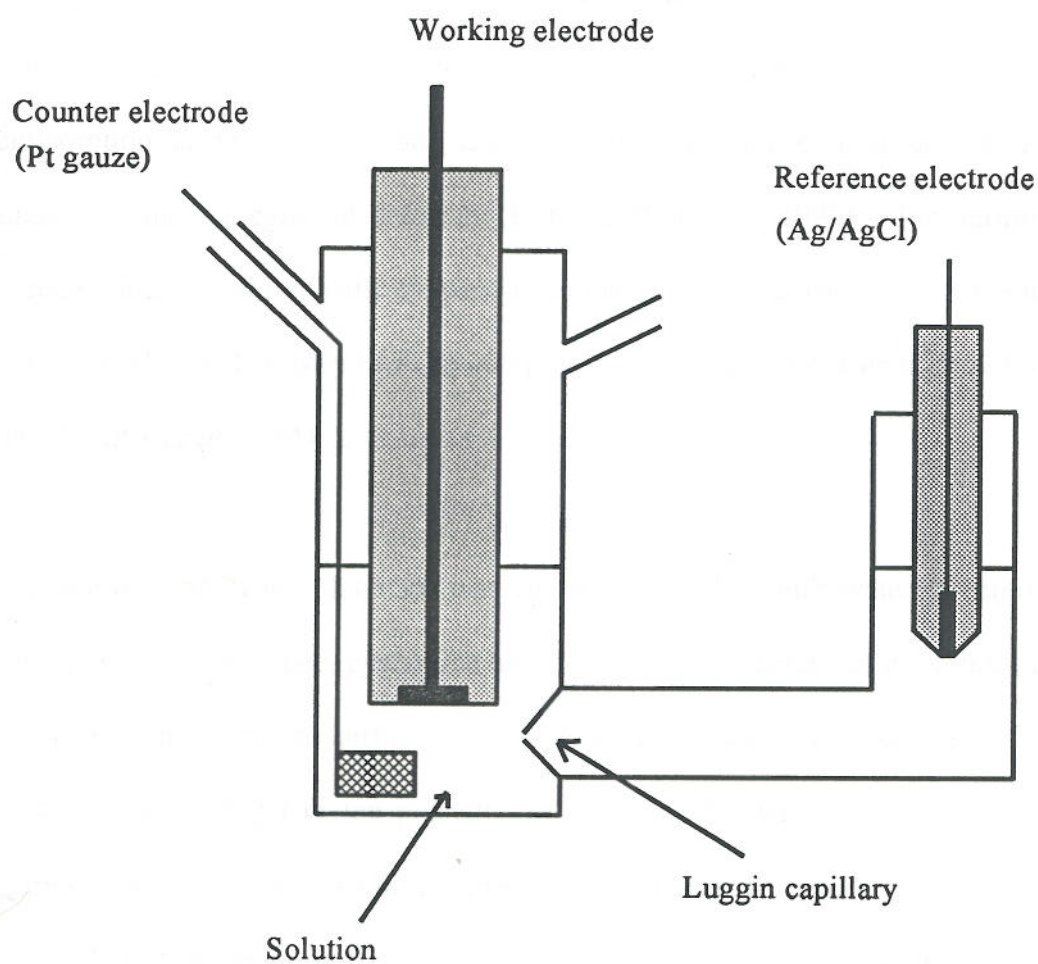


Figure 4.1 Two-compartment electrochemical cell for stationary electrode voltammetry.

Cyclic voltammograms were recorded at pH 2.0, 3.5, 4.0, 5.0, 6.0 and 7.0 before and after the addition of 50 μ M glucose oxidase to the working compartment.

4.2.6 Development of PQQ-modified enzyme electrodes

A Pt foil electrode (geometrical area 0.283 cm²) was soaked in a solution of 0.02 M cystamine (2,2'-diaminodiethyldisulfide (Aldrich) in water for 2 h. Cystamine chemisorption at the Pt electrode decreases the capacity current during potential scanning in the background solution (Katz & Solov'ev, 1992). Furthermore this electrode functionalisation with cystamine amino groups has been recently shown to enhance the electrochemistry of PQQ and provide the basis for covalent immobilization of PQQ (Katz *et al.*, 1994a).

The cystamine-modified electrode was rinsed thoroughly with water to remove any excess, physically adsorbed, cystamine and then used to generate an immobilized PQQ monolayer at the electrode surface. This was achieved via incubation for 2 h in a 3 mM solution of PQQ in the presence of 10 mM of a water-soluble carbodiimide coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma), in 0.01 M HEPES aqueous buffer (pH 7.5). The PQQ-modified electrode was rinsed thoroughly with water to remove the non-immobilised physically adsorbed PQQ. The PQQ-modified cystamine electrode was then incubated for 12 hours in the presence of 400 μ M glucose oxidase at 4°C.

After incubation with glucose oxidase, 10 μ l of a 10 % solution of glutaraldehyde was

added to the surface of the PQQ-modified cystamine electrode and allowed to air dry for 90 min in a fume cupboard at 25°C. The source of glutaraldehyde was Sigma Type I, supplied as a 25 % solution which was stored frozen and diluted to 10 % in distilled water immediately before use (Cass, 1990). The electrodes were then rinsed in distilled water prior to amperometric detection of glucose.

4.2.7 Amperometric detection of glucose in fruit juice

The pH of several fruit juices was determined before assay in order to assess their compatibility for determination of glucose at a glucose oxidase-PQQ modified enzyme electrode at pH 3.5. Consequently, Libby's tomato juice (Nestlé U.K. Ltd., Croydon, Surrey) and RibenaTM black currant juice drink (Smithkline Beecham Consumer Brands, Brentford, U.K.) were both chosen for determination of glucose. The pH of the tomato juice was determined as 4.0 and that of the RibenaTM was 3.2.

Amperometry was performed using an Autolab Electrochemical analyser (Windsor Scientific, Windsor, U.K.) as previously described in section 2.2.2. The enzyme electrodes were immersed in 10 ml of 0.1 M citrate buffer, pH 3.5 (containing 0.1 M KCl supporting electrolyte) contained in a 20 ml glass water-jacketed cell, (Soham Scientific, Ely, Cambs.) thermostated at 25°C using a circulating water bath (Gallenkamp, Loughborough, Leics.).

Glucose oxidase-PQQ modified enzyme electrodes were poised at +300 mV vs an Ag/AgCl reference electrode (Biotech. Instruments, Luton, U.K.) for amperometric

detection of glucose substrate in the presence of a platinum gauze auxiliary electrode. The current detected on addition of 5 mM aliquots of glucose provided a calibration curve (Figure 4.10a) for the amperometric determination of glucose in 50 μ l aliquots of RibenaTM and tomato juice at PQQ-modified glucose oxidase enzyme electrodes.

4.2.8 UV-Determination of glucose in fruit juice

A commercial test kit was purchased from Boehringer Mannheim Diagnostics Ltd. (East Sussex, U.K.), and used in accordance with the manufacturer's instructions for the determination of D-glucose in fruit juice. The glucose concentration determined by the standard UV method was compared with results determined amperometrically to enable the accuracy of glucose measurements in fruit juice, using a PQQ-modified glucose oxidase electrode to be assessed.

The test kit combination contained :

Reagent 1: a 7.2 g powder mixture consisting of: triethanolamine buffer, pH 7.6; NADP, 110 mg; ATP, 260 mg; magnesium sulphate; stabilizers. This reagent was dissolved in 45 ml distilled water immediately prior to the enzymatic determination of glucose.

Reagent 2: enzyme suspension, 1.1 ml, consisting of: hexokinase, 320 U; glucose-6-phosphate dehydrogenase, 160 U. This reagent was used undiluted.

Reagent 3: standard glucose solution (0.5 g/litre). This reagent was diluted 1:10 with distilled water to provide a standard glucose solution containing 0.05 g/litre which was then used to prepare sample solution containing 5-25 μ g of D-glucose per cuvette (in

0.1-2.0 ml sample volume) in order to obtain a calibration curve for subsequent determination of glucose in fruit juice.

Assay solutions were prepared as shown in Table 4.1 to enable UV determination of 5-25 μg glucose in 3 ml cuvettes at 340 nm using a CECIL CE 594 UV-Visible Spectrophotometer (CECIL Instruments Ltd., Cambridge).

Absorbance readings were taken at 25°C against a blank cuvette which contained **Reagent 1** (Tris buffer/NADP pH 7.6) and distilled water. Each assay (1-10 inc) was prepared in triplicate to provide a total of 30 solutions for absorbance measurement. The sample solution, distilled water and **Reagent 1** (Tris buffer/NADP pH 7.6) were added to each cuvette. The contents of each cuvette were mixed and the initial absorbance (A_1) was read after 3 min. The enzymatic assay was then started by the addition of 0.02 ml of **Reagent 2** (enzyme suspension) to each cuvette. Contents were mixed and the absorbances of the solutions (A_2) were read at the end of the enzyme reaction (after 15 min).

The $\Delta A_{\text{D-glucose}}$ was then determined for each sample by subtracting the absorbance difference of the blank ($A_1 - A_2$) from the absorbance difference of the sample. The mean absorbance of each glucose standard ($n=3$) provided a calibration curve (Figure 4.11) for UV-determination of glucose in Ribena™.

Triplicate cuvettes were also prepared for both Ribena™ and tomato juice which

contained the respective fruit juice and all assay reagents except **Reagent 2** (enzyme solution). These cuvettes provided sample blanks to account for any background absorbance arising from the fruit juice and were also used to establish the presence of any interferents in each fruit juice which might react with the NADP in the absence of enzyme suspension.

Solution	Contents	Sample volume	Distilled water
1	BLANK	0.00 ml	2.00 ml
2	GLUCOSE	0.10 ml	1.90 ml
3	GLUCOSE	0.20 ml	1.80 ml
4	GLUCOSE	0.30 ml	1.70 ml
5	GLUCOSE	0.40 ml	1.60 ml
6	GLUCOSE	0.50 ml	1.50 ml
7	RIBENA	0.10 ml	1.90 ml
8	RIBENA	0.20 ml	1.80 ml
9	TOMATO	0.10 ml	1.90 ml
10	TOMATO	0.20 ml	1.80 ml

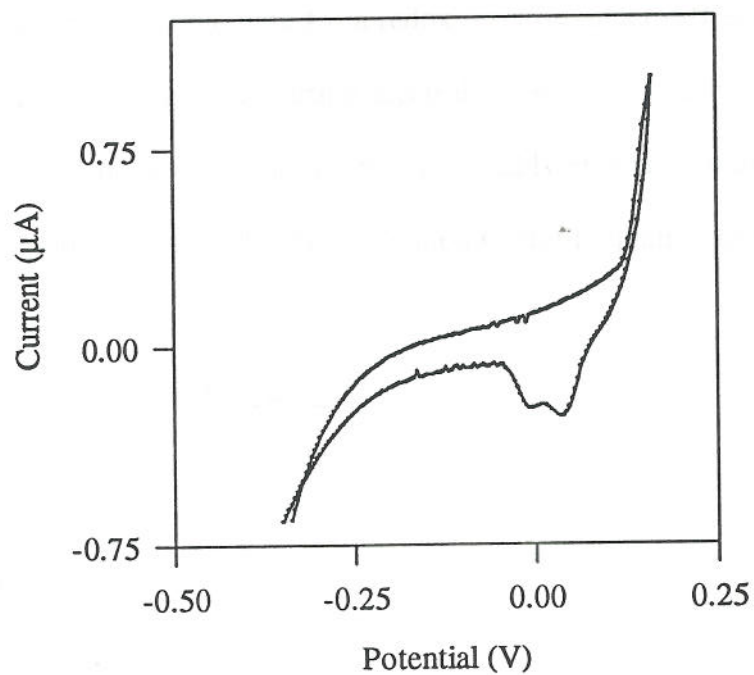
Table 4.1 Preparation of assay solutions for UV-determination of glucose in fruit juice. Glucose standard contained 0.05 g glucose/litre. RibenaTM and tomato juice were diluted 1:200 before addition of 0.1 ml and 0.2 ml aliquots to the respective sample solutions.

4.3 RESULTS AND DISCUSSION

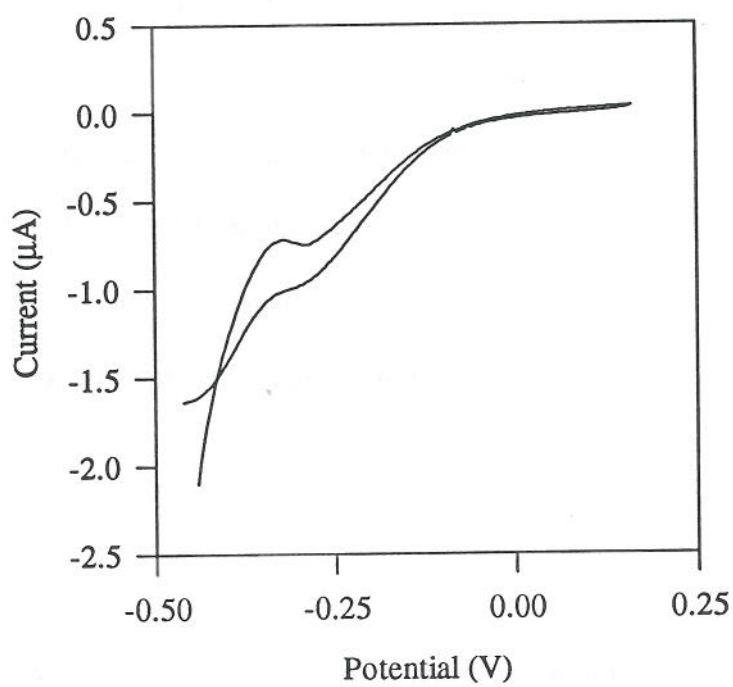
4.3.1 Electrochemistry of PQQ

The electrochemistry of a solution containing 3 mM PQQ in 0.1 M phosphate buffer (pH 7.0) was investigated at different metal electrodes. In the case of a gold electrode (Figure 4.2a) a small reduction peak was observed at +0.032 V with a shoulder at -0.01 V. However no oxidation peak was observed. No obvious redox peaks were observed during cyclic voltammetry of 3 mM PQQ at an unmodified platinum electrode (Figure 4.2 b).

The absence of distinct peak shaped voltammograms observed in Figure 4.2 indicates that electron transfer between PQQ and either gold or platinum electrodes is difficult. The platinum electrode was subsequently coated with a polypyrrole membrane in order to facilitate electron transfer between PQQ and the electrode surface in accordance with previous work by Shinohara *et al.* (1991). This could then provide a possible route for the development of PQQ modified electrodes.



(a)



(b)

Figure 4.2. Cyclic voltammograms of PQQ with (a) gold and (b) platinum electrodes in a 0.1 M phosphate buffer solution (pH 7.0, 0.1 M KCl electrolyte). PQQ concentration 3 mM, $\nu = 5 \text{ mV.s}^{-1}$. Potentials reported vs S.C.E. reference electrode.

4.3.2 Determination of the electrochemical area of electrodes

When charge is measured against time for a redox reaction under diffusion control (an un-stirred solution containing a supporting electrolyte, with the electrode poised at a potential where the rate of electron transfer is considerably greater than the rate of mass transport), Q can be related to $t^{1/2}$ by the integrated Cottrell equation (Anson, 1967):

$$Q = \frac{2nFAD^{1/2}c^b t^{1/2}}{\pi^{1/2}}$$

where

Q = Charge at time t

n = Number of electrons

F = Faraday Constant ($96,485 \text{ C.mol}^{-1}$)

A = Electrode area (cm^2)

c^b = Concentration of redox species in bulk solution

($\text{K}_4\text{Fe}(\text{CN})_6$, $1 \text{ mM} \equiv 1 \times 10^{-6} \text{ mol.cm}^{-3}$)

D = Diffusion coefficient of redox species

($\text{K}_4\text{Fe}(\text{CN})_6$, $6.3 \times 10^{-6} \text{ cm}^2.\text{s}^{-1}$, Adams, 1969)

$\pi = 3.1412$

t = time (s)

Hence a plot of Q as a function of $t^{1/2}$ is linear and gives a gradient of

$$\text{Gradient} = \frac{2n FAD^{1/2}c^b}{\pi^{1/2}}$$

Therefore for a reaction where $n = 1$, the electrochemical surface area (A) can be given by

$$A = \frac{(\text{Gradient})\pi^{1/2}}{2F(D)^{1/2}c^b}$$

The electrochemical surface area in these calculations was determined by chronocoulometric measurements of a solution of 1 mM $K_4Fe(CN)_6$, assuming a diffusion coefficient for the redox couple of $6.3 \times 10^{-6} \text{ cm}^2.\text{s}^{-1}$ (Adams, 1969).

Figure 4.3 illustrates a typical plot of Q vs. $t^{1/2}$ from which the electrochemical area of a platinum foil electrode was determined in accordance with the integrated Cottrell equation.

The mean electrochemical area of three Pt foil working electrodes studied ($0.313 \text{ cm}^2 \pm 0.022$) was greater than the area determined geometrically (0.283 cm^2 , $r = 0.3 \text{ cm}$). The reason for this is because the electrodes have a microscopically rough surface which is porous, hence the real surface area will be three-dimensional. Geometric measurements only provide a two-dimensional estimate of the apparent surface area. The corresponding ratio of geometric area to real surface area is called the roughness factor and for solid electrodes is often greater than unity (Plambeck, 1982). The roughness factor for the Pt foil electrodes was determined to be 1.11.

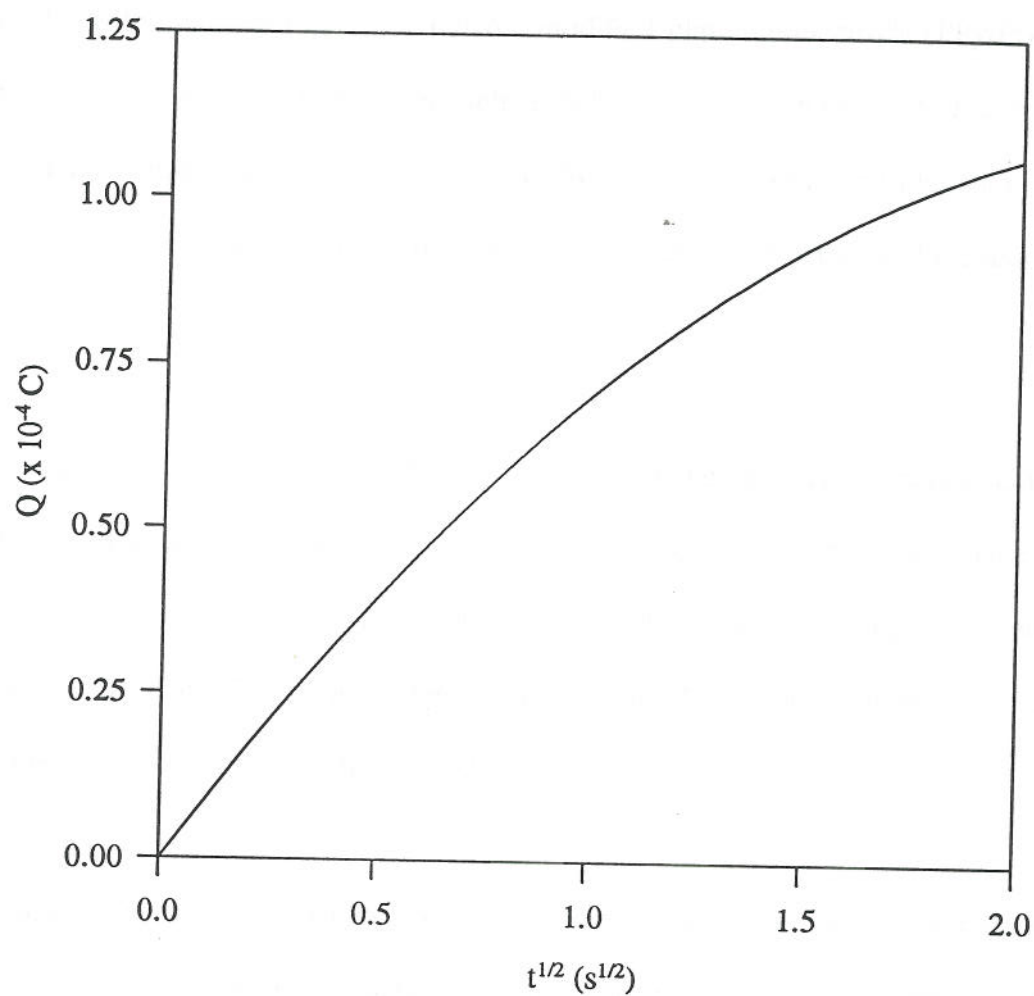


Figure 4.3. Typical plot (Q vs. $t^{1/2}$) for determination of electrochemical area of a platinum foil electrode. Electrode poised at +450 mV vs. SCE in 1 mM $K_4Fe(CN)_6$ for 2 seconds (pulse 0.01 sec); Gradient $9.074 \times 10^{-5} Q.s^{-1/2}$; Correlation coefficient 0.994.

4.3.3 Electrochemistry of PQQ at polypyrrole-modified electrodes

The cyclic voltammogram of a polypyrrole modified platinum electrode (PPy/Pt) in a solution containing 3 mM PQQ in phosphate buffer of pH 7.0 is shown in Figure 4.4. The oxidation peak observed at -0.116 V (vs SCE) and corresponding reduction peak at -0.225 V (vs SCE) were attributed to the electrochemical oxidation and reduction of PQQ.

Close inspection of the cyclic voltammogram, (peak height, peak separation, peak potential) and analysis of the effect of increasing scan rate on the peak shape and distribution, enable the electron transfer between PQQ and the PPy modified Pt electrode to be classified in accordance with specified criteria for reversible, quasi reversible and irreversible electron transfer.

The oxidation peak current ($0.06 \mu\text{A}$) was almost the same as that of the reduction ($0.068 \mu\text{A}$), and the relationship between scan rate and oxidation peak current (Figure 4.5) shows that the oxidation peak current was proportional to the square root of the scan rate. This result indicates that the electron transfer between PQQ and PPy-modified electrodes is reversible and diffusion controlled.

However, the observed peak separation (Figure 4.4) was 109 mV which is much greater than the theoretical peak separation quoted for reversible electron transfer (i.e., $57/n$ mV where n is the number of electrons transferred (Davis, 1985; Hall 1990)). Since the electrochemical oxidation and reduction of PQQ at a polypyrrole membrane electrode

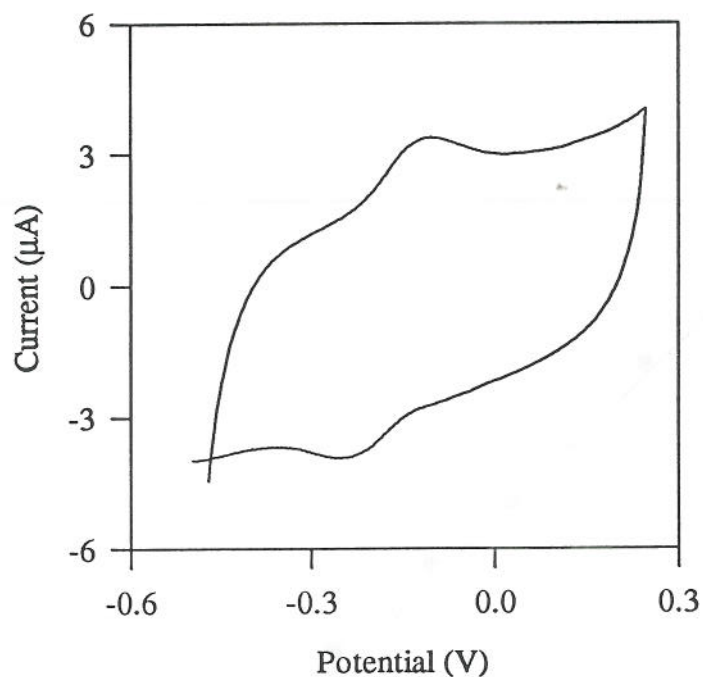


Figure 4.4. Cyclic voltammogram of PQQ with a polypyrrole modified platinum electrode (polymerisation charge 100 mC.cm^{-2}) in a 0.1 M phosphate buffer solution ($\text{pH } 7.0$, 0.1 M KCl electrolyte). PQQ concentration 3 mM , $\nu = 5 \text{ mV.s}^{-1}$. Potentials recorded vs S.C.E. reference electrode.

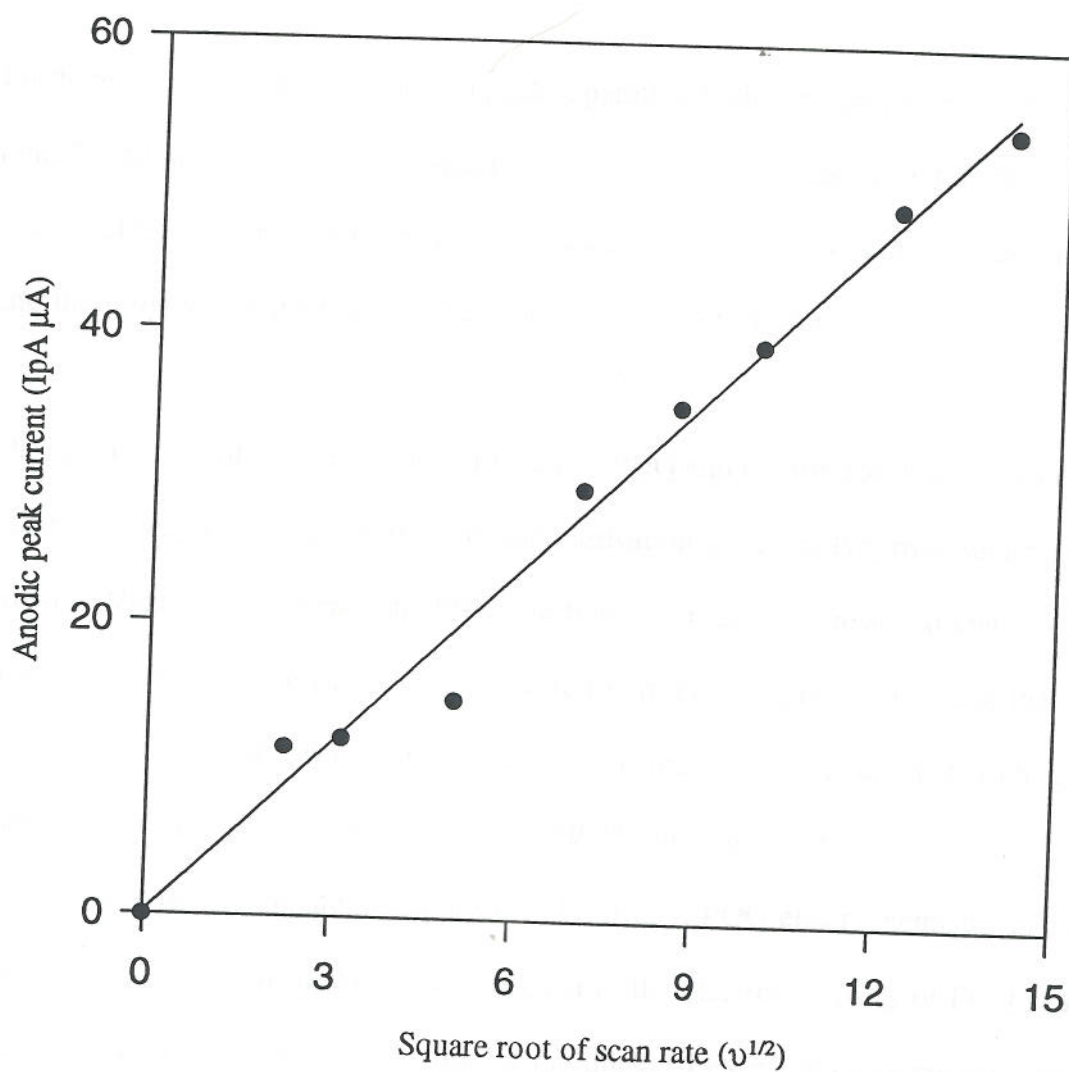


Figure 4.5 Relationship between square root of scan rate and anodic peak current for a polypyrrole modified platinum electrode (polymerisation charge 100 mC.cm⁻²) in 0.1 M phosphate buffer, pH 7.0, containing 3 mM PQQ.

has previously been characterised as a one-step two electron transfer process (Shinohara *et al.*, 1991), for reversible electron transfer of PQQ in this system the theoretical peak separation should be 28.5 mV.

Furthermore, the peak potential and peak separation both increased with increasing scan rate. These observations with respect to the size of anodic peak current, peak separation and the effect of increasing scan rate all indicate that the electron transfer between PQQ and the polypyrrole membrane electrode is "quasi" reversible.

The quasi reversible electron transfer between PQQ and the surface of the PPy modified electrode may be due to inefficient polymerisation of the polypyrrole and poor film growth. Visual inspection of the PPy modified electrode often revealed that the Pt disk electrode was not always completely covered with PPy despite the fact that the charge passed, 100 mC.cm², was equivalent to that required for optimal film growth (Shinohara *et al.*, 1991). Thicker films 200/400 mC.cm² where complete coverage of Pt disc was visually apparent displayed little, if any, PQQ electrochemistry indicating the importance of film thickness with respect to the electrochemistry of PQQ at a PPy modified electrode. Closer inspection of the thicker PPy membranes revealed uneven, pitted film growth at the electrode surface.

Possible alternatives for future investigation may involve polymerisation of pyrrole from organic solvent (e.g. acetonitrile) as opposed to aqueous 0.1 M KCl. In this respect Dicks *et al.* (1989) found that the structure of PPy films formed in aqueous electrolyte

were more coarse in appearance with a less homogeneously packed structure than PPy films formed in acetonitrile. However, Diaz (1981) previously reported that PPy films grown in anhydrous acetonitrile have a rough surface with dendrite-like structures whereas addition of as little as 1 % water to the solvent enabled growth of much smoother PPy films at the electrode surface (Diaz, 1981).

4.3.4 Cyclic voltammetry of glucose oxidase and PQQ

For the duration of the cyclic voltammograms observed on addition of 50 μM glucose oxidase to a solution containing 3 mM PQQ and 50 mM glucose at different pH values (Figures 4.6 - 4.8 respectively) it is important to consider that: PQQ was supplied in the oxidised form; the enzyme was predominantly in the reduced form due to the large excess of glucose, i.e., $[\text{glucose}]/K_M > 2$ where K_M is the Michaelis constant for glucose and is equal to 20 mM (Gibson *et al.*, 1964; Swoboda & Massey, 1965).

Consequently if electron transfer occurs between the reduced FAD moiety of glucose oxidase and oxidized PQQ, structural interaction should produce sigmoidal catalytic currents in accordance with the following equations:



Figure 4.6a shows that there was little change to the cyclic voltammogram on addition of 50 μ M glucose oxidase to a solution of 3 mM PQQ and 50 mM glucose in 0.1 M citrate buffer at pH 2.0. Since glucose oxidase has an isoelectric point of 4.2 (Swododa & Massey 1965; Tsuge et al., 1984), at pH 2.0 the enzyme has a significant net positive charge. In addition, PQQ becomes protonated at pH $\leq$ 5 (Boeriu *et al.*, 1993; 1994). It is therefore reasonable to assume that electrostatic repulsion prevents electron transfer between PQQ and the FAD moiety of glucose oxidase at pH 2.0.

However, a significant increase in anodic current (0.165 μ A) and corresponding decrease in the cathodic current was evident following the addition of glucose oxidase to a solution of PQQ and glucose in 0.1 M citrate buffer at pH 3.5 (Figure 4.6b). Although this pH is slightly less than the isoelectric point of glucose oxidase the enzyme has no positive charge which would otherwise repel the protonated PQQ.

A catalytic current (0.15 μ A) was also observed at pH 4.0 (Figure 4.7a) and although the maximum catalytic current (0.18 μ A) was observed at pH 5.0 (Figure 4.7b) this was accompanied by a large shift in peak potential (+500 mV). This shift in catalytic peak potential at pH 5.0 was not observed during similar coupling at pH 3.5 and pH 4.0 (Figure 4.6b & Figure 4.7a).

Since PQQ is known to have a pKa of 5.0 (Boeriu *et al.*, 1993; Kano *et al.*, 1990a) it is possible that the protonation of PQQ could contribute to the large current and peak shift observed at this pH (Figure 4.6b).

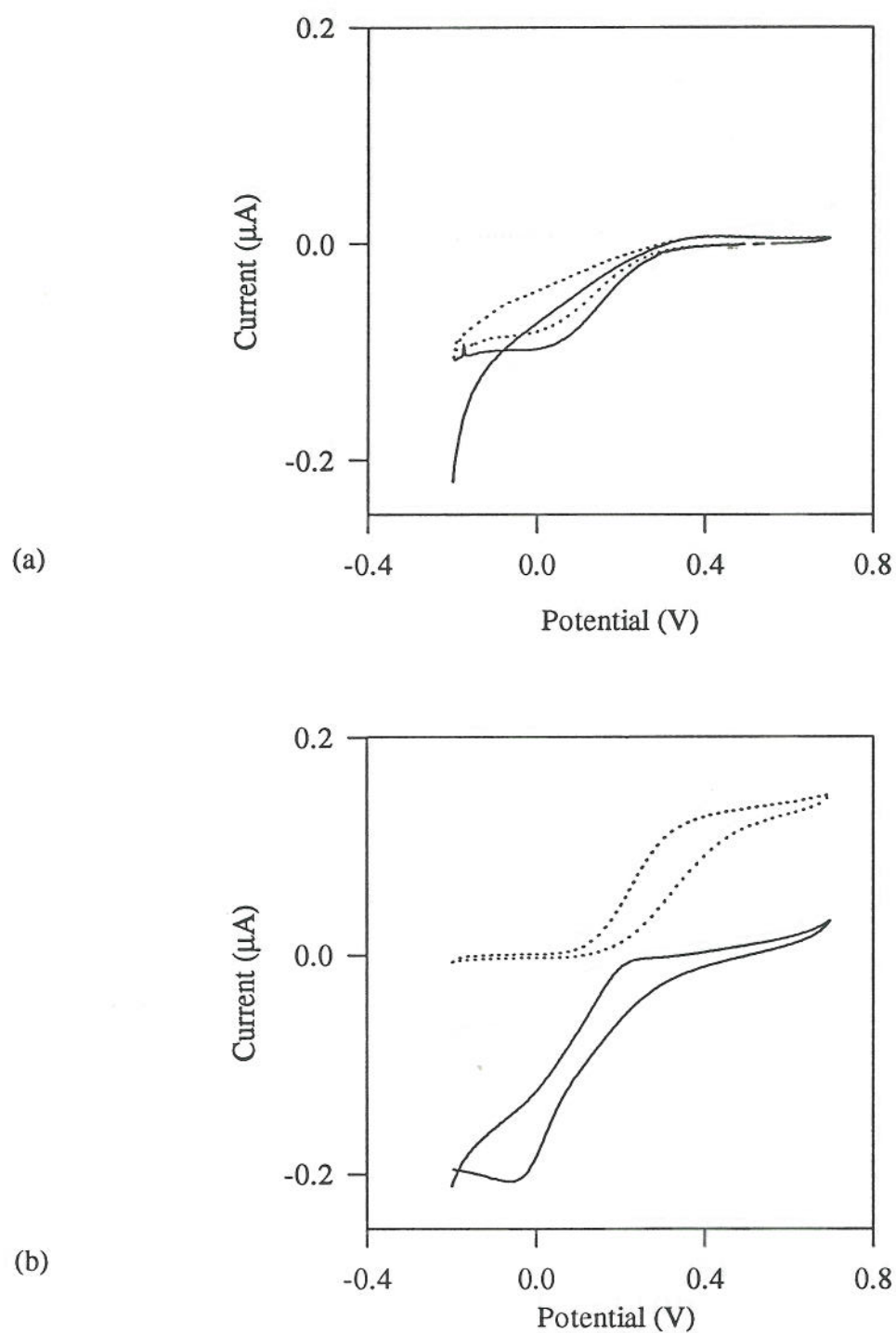


Figure 4.6. Cyclic voltammograms of PQQ before (—) and after (....) addition of 50 μM glucose oxidase in 0.1 M citrate buffer at (a) pH 2.0, (b) pH 3.5. Buffer contained 3 mM PQQ and 50 mM glucose. Potentials reported vs. Ag/AgCl reference electrode. Scan rate = 5 mV.s^{-1} .

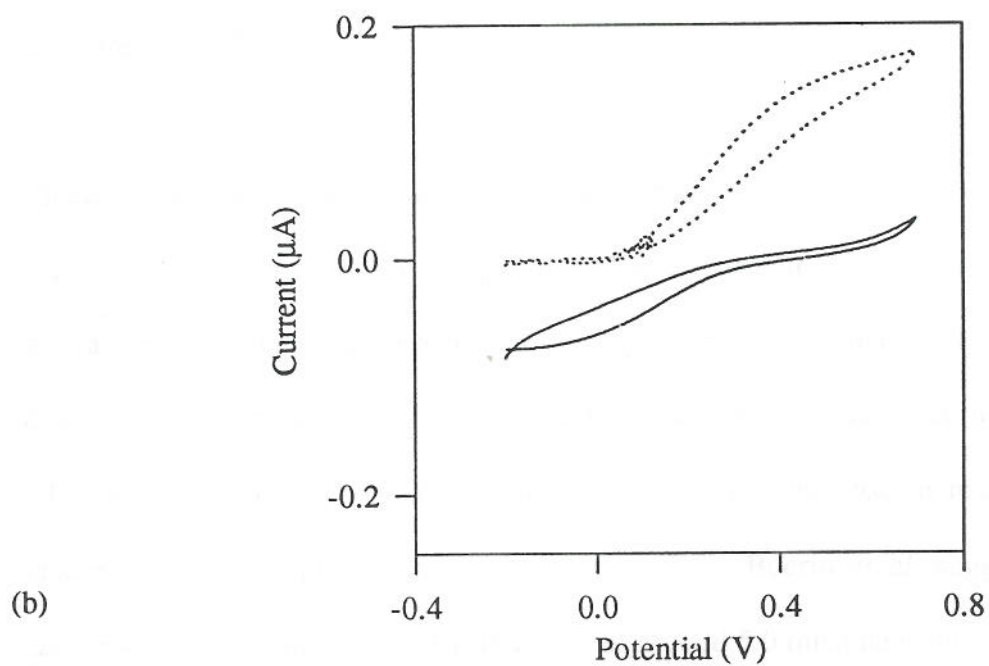
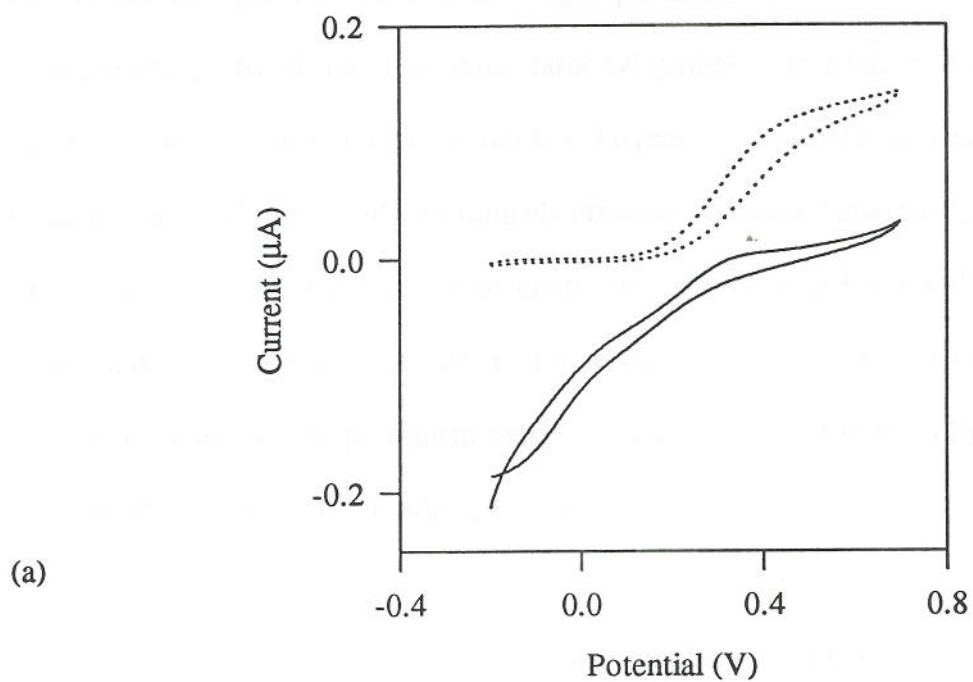


Figure 4.7. Cyclic voltammograms of PQQ before (—) and (....) after addition of $50\text{ }\mu\text{M}$ glucose oxidase in 0.1 M citrate buffer at (a) pH 4.0, (b) pH 5.0. Buffer contained 3 mM PQQ and 50 mM glucose. Potentials reported vs Ag/AgCl reference electrode.

Scan rate = $5\text{ mV}\cdot\text{s}^{-1}$.

At pH 6.0 and pH 7.0, PQQ is no longer protonated and carries a significant negative charge by virtue of its three carboxyl groups. In addition, the net negative charge on the surface of glucose oxidase increases as the pH increases beyond the isoelectric point. Consequently a strong electrostatic repulsion between PQQ and glucose oxidase accounts for the absence of coupling observed at pH 6.0 and pH 7.0. This electrostatic repulsion may be sufficient to destabilise the reduced PQQ as indicated by the disappearance of the prominent reduction peak currents of PQQ in Figures 4.8a and 4.8b, at pH 6.0 and pH 7.0, after addition of glucose oxidase.

The results presented in Figures 4.6 - 4.8 emphasize that a pH less than 6.0, is necessary for interaction between PQQ and glucose oxidase to occur and that interaction is optimal at pH 3.5.

These results are consistent with a recent report by Boeriu *et al.* (1993) who investigated the use of PQQ as a possible electron acceptor for glucose oxidase in the pH range 2.4 - 8.0. Using spectrophotometry, Boeriu *et al.* independently established that no reaction between PQQ and glucose oxidase could be detected in the pH range 6.0 - 8.0 (Boeriu *et al.* 1993). However, a three-fold increase in reaction rate was observed when the pH decreased from 5.0 to 3.0. Boeriu *et al.* suggested that the carboxylic acid groups of PQQ with a pKa of around 5.0 must be protonated before the appropriate structural interaction between the quinone moiety of PQQ and the FAD moiety of GOD can occur. The resultant charge transfer complex formation facilitates electron transfer from the reduced FAD moiety of glucose oxidase to PQQ prior to

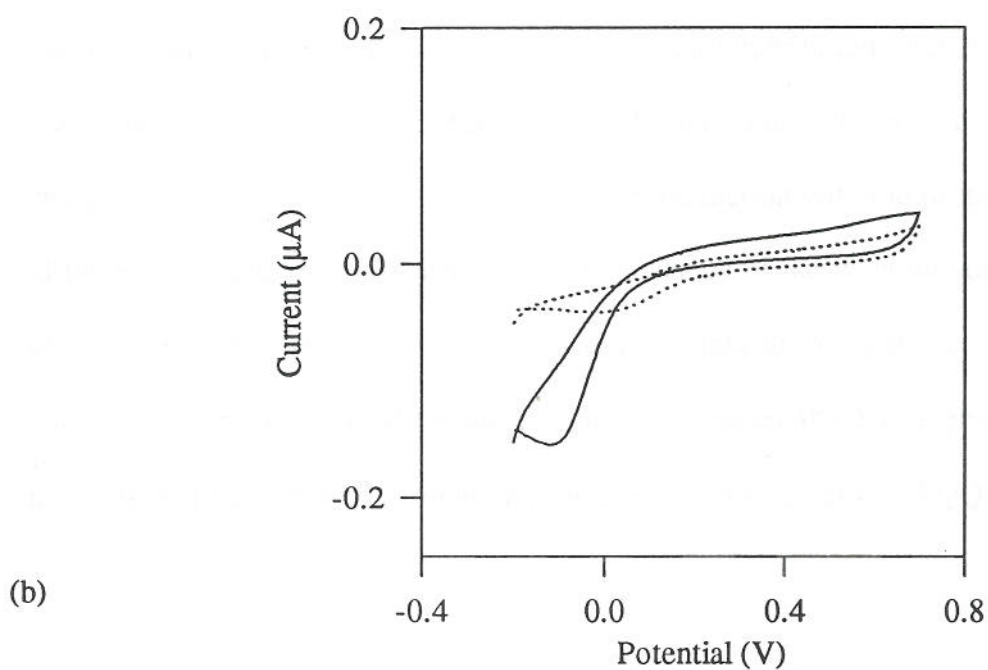
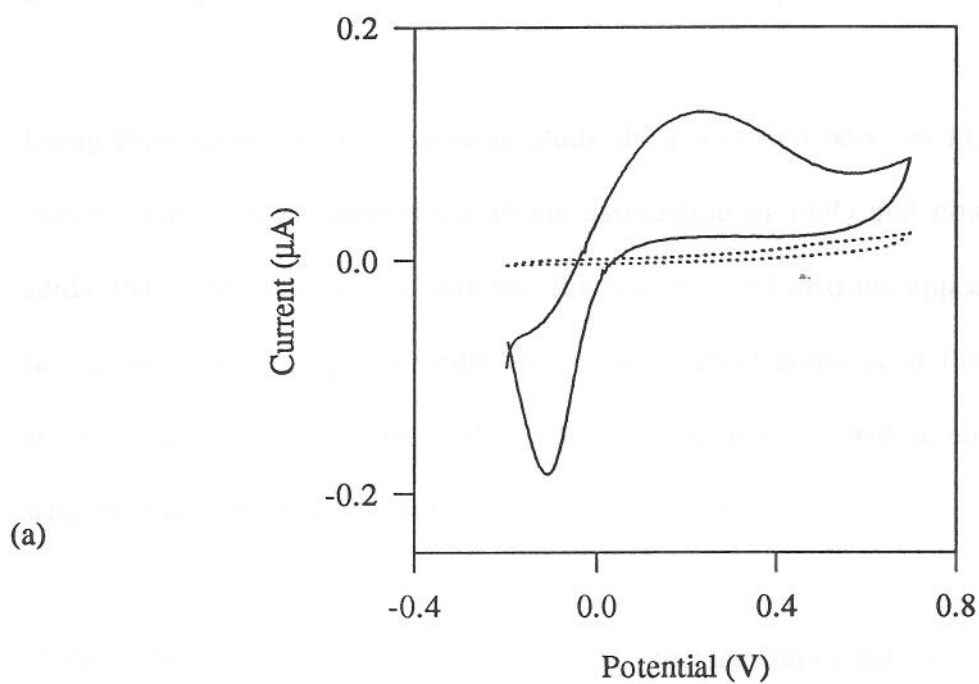


Figure 4.8. Cyclic voltammograms of PQQ before (—) and (....) after addition of $50\ \mu\text{M}$ glucose oxidase in $0.1\ \text{M}$ citrate buffer at (a) pH 6.0, (b) pH 7.0. Buffer contained $3\ \text{mM}$ PQQ and $50\ \text{mM}$ glucose. Potentials reported vs Ag/AgCl reference electrode. Scan rate = $5\ \text{mV}\cdot\text{s}^{-1}$.

glucose catalysis.

Using fluorescence measurements to study the interaction between PQQ and glucose oxidase Boeriu *et al.* observed a strong association of PQQ and glucose oxidase at acidic pH. The quenching of quinone fluorescence and also the appearance of a new fluorescence was thought to result from non-covalent contacts of PQQ with several amino acid side chains of the FAD binding site of glucose oxidase and complexation with the flavin moiety of FAD (Boeriu *et al.*, 1993).

More recently, Boeriu *et al.* suggested that the mechanism of the reduction of PQQ by glucose oxidase may involve a reactive *p*-quinoid structure of PQQ. This *p*-quinoid tautomer was thought to be due to a keto-enol tautomerization of PQQ on interaction with glucose oxidase (Boeriu *et al.*, 1994). In addition to the *p*-quinoid structure the charge of PQQ was also reported to affect the interaction with the protein environment of the enzyme and also influence the pH dependency of the reaction between PQQ and glucose oxidase. However since there is little data to confirm the existence of the reactive *p*-quinoid intermediate the interaction between PQQ and glucose oxidase is more likely to concern the protonation of the carboxyl groups of PQQ at $\text{pH} \leq 5.0$.

4.3.5 Cyclic voltammetry of PPy-PQQ modified electrodes with glucose oxidase

Voltammetric analysis of a PPy-PQQ modified electrode at pH 7.0 containing 200 μ M glucose oxidase showed no catalytic coupling on addition of 50 mM glucose substrate (Figure 4.9). The oxidation peak at -200 mV was slightly reduced on addition of glucose suggesting that a PPy-modified electrode with entrapped 3 mM PQQ and 200 μ M glucose oxidase was unstable.

Amperometric analysis of similar PPy modified electrodes with entrapped 200 μ M glucose oxidase and 3 mM PQQ in 0.1 M phosphate at pH 7.0 revealed no response to 10 mM aliquots of glucose at -110 mV vs Ag/AgCl. These results indicate that a PPy-PQQ-modified electrode does not facilitate electron transfer from glucose oxidase at pH 7.0.

Cyclic voltammetry of a PPy modified electrode with entrapped 3 mM PQQ in 0.1 M citrate buffer pH 3.5 showed a significant increase in anodic peak current on addition of 50 mM glucose, in the absence of glucose oxidase (Figure 4.10). The gradual red colouration of the surrounding buffer observed during this voltammetry was attributed to the leaching of PQQ as glucose was oxidized directly at the PPy membrane. Subsequent addition of 200 μ M glucose oxidase did not significantly alter the previous voltammogram obtained after addition of glucose.

The inability to couple with glucose oxidase, the inefficient polymerisation of polypyrrole and the "quasi" reversible electron transfer of PQQ observed at PPy

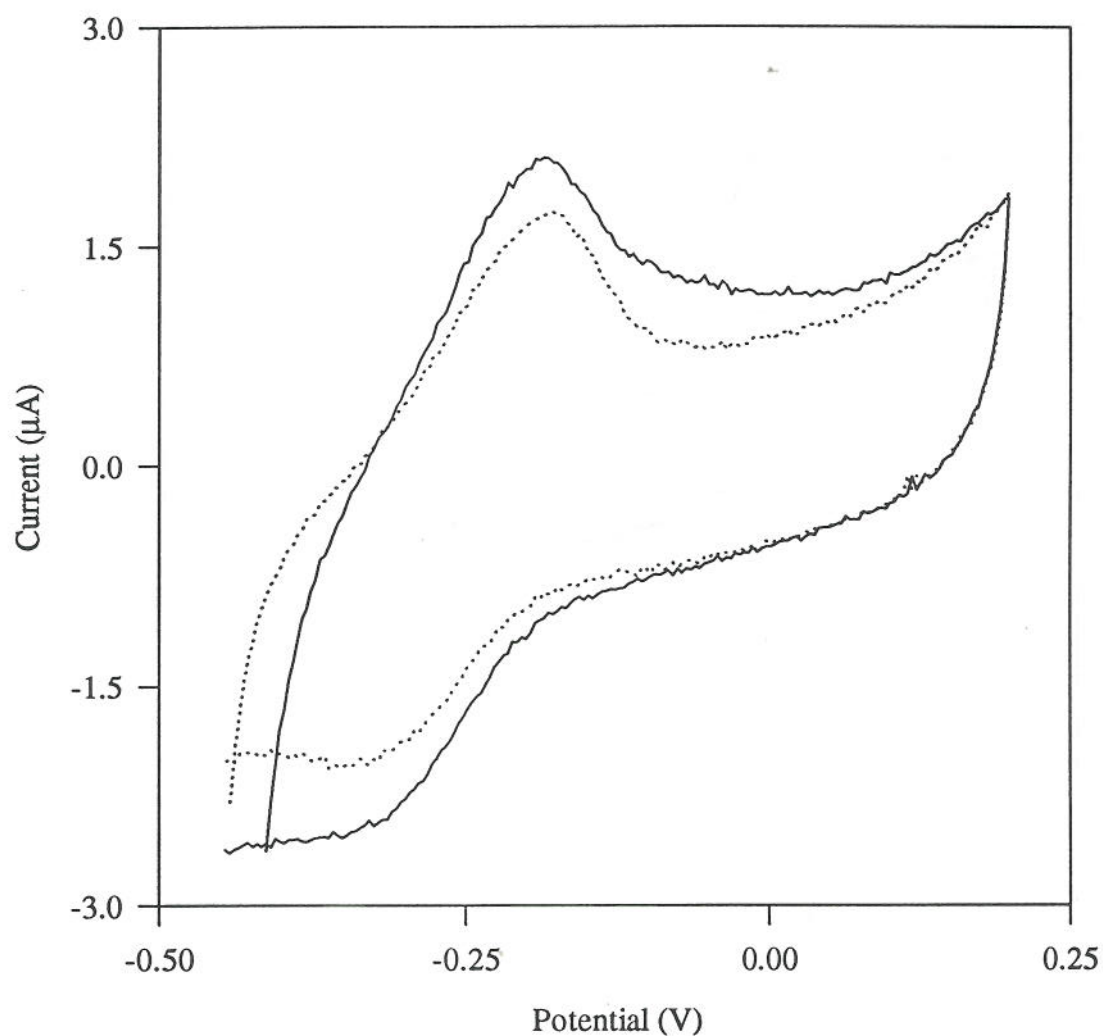


Figure 4.9. Cyclic voltammograms of PPy-PQQ modified electrode with 200 μM glucose oxidase before (—) and after (....) addition of 50 mM glucose in 0.1 M phosphate buffer, pH 7.0. Polymerisation charge 100 $\text{mC}\cdot\text{cm}^{-2}$, PQQ concentration 3 mM, $\nu = 5 \text{ mV}\cdot\text{s}^{-1}$.

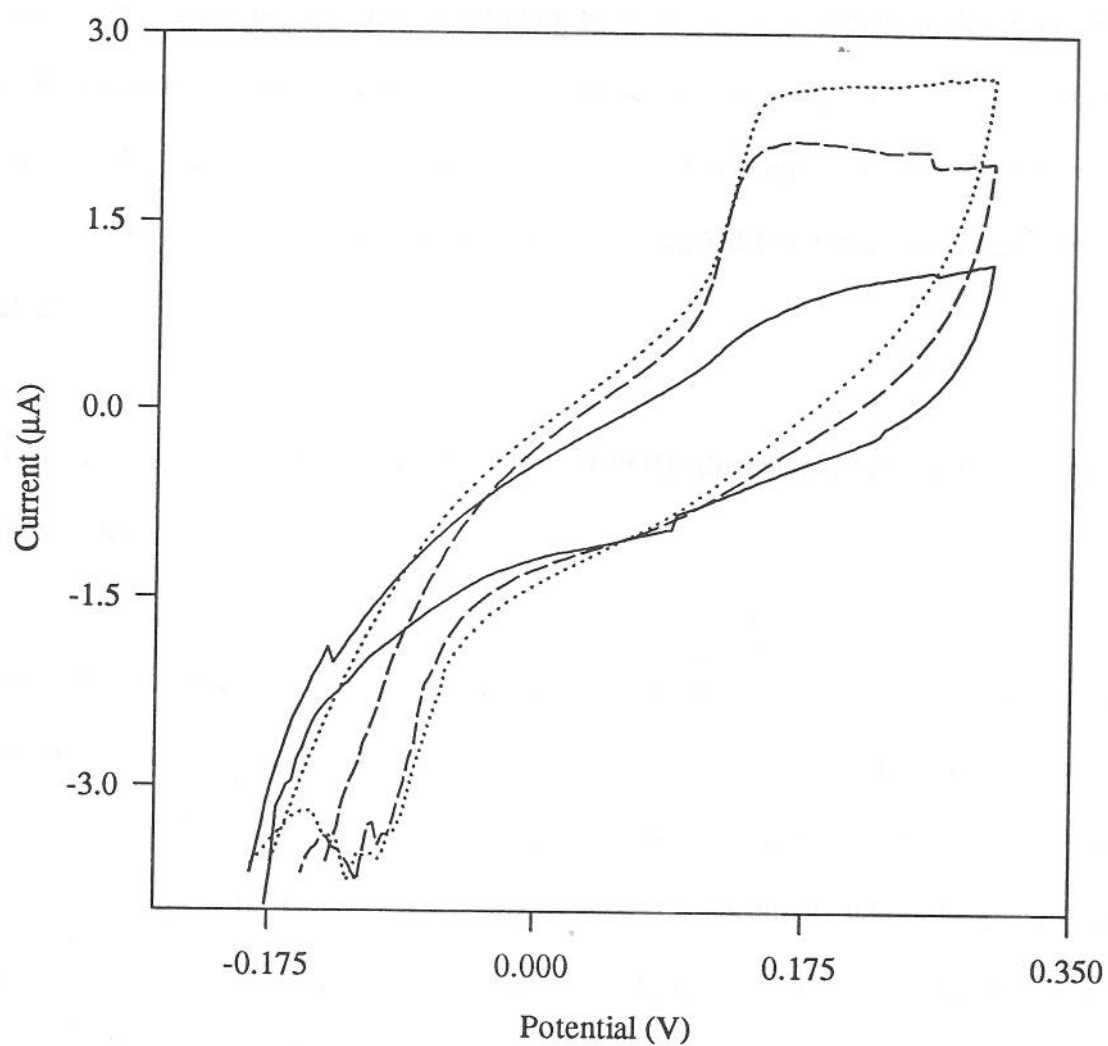


Figure 4.10. Cyclic voltammograms of PPy-PQQ modified electrode, without glucose oxidase before (—) and after (---) addition of 50 mM glucose in 0.1 M citrate buffer pH 3.5. (.....) represents cyclic voltammogram observed after subsequent addition of 200 μ M glucose oxidase. Polymerisation charge 100 mC.cm^{-2} , PQQ concentration 3 mM, $\nu = 5 \text{ mV.s}^{-1}$.

modified electrodes indicate that PPy membrane electrodes are not suitable for future development of PQQ modified enzyme electrodes.

Therefore cystamine modified electrodes were used for subsequent development of PQQ-glucose oxidase modified electrodes in accordance with the methods of Katz *et al.*, (1994 a,b,c). Accordingly the results presented in sections 4.3.6 and section 4.3.7 relate to the detection of glucose at cystamine modified PQQ-glucose oxidase enzyme electrodes.

4.3.6 Amperometric detection of glucose at PQQ-glucose oxidase modified enzyme electrodes

To ensure the accuracy of any glucose enzyme electrode the effects of direct glucose oxidation must be appreciated. Failure to consider this point may obscure any detrimental effects incurred when investigating new methods of enzyme immobilisation (White *et al.*, 1994). Consequently, amperometric experiments without PQQ and then without enzyme established the absence of direct glucose oxidation at the Pt electrode surface, and verified the requirement of both PQQ and glucose oxidase for the amperometric response to glucose.

4.3.7 Amperometric detection of glucose in fruit juice

4.3.7.1 Detection of glucose in tomato juice

It should be emphasized that glucose could not be detected in tomato juice either

amperometrically nor using a commercial test kit supplied by Boehringer Mannheim. The total amount of sugar present in Libby's tomato juice was less than 3.1 g as stated in the ingredients, and could not be detected. In addition to the fact that all this sugar is not necessarily glucose one would also expect to find sucrose a natural sugar of all fruits, the heterogeneous nature of the tomato juice also prevented detection of the low sugar levels (< 3.1 g/100 ml).

4.3.7.2 Detection of glucose in Ribena™

The current response observed on addition of glucose to PQQ-glucose oxidase modified enzyme electrodes (Figure 4.11a) was linear between 0 and 5 mM glucose before reaching saturation at glucose concentrations greater than 10 mM.

The current response observed on addition of Ribena™ to PQQ-glucose oxidase modified enzyme electrodes (Figure 4.11b) was linear in the range 0 - 0.18 μ A. This corresponded to the addition of 0 - 300 μ l undiluted aliquots of Ribena™ to 10 ml of 0.1 M citrate buffer, pH 3.5 at 25°C.

Comparison of the current output associated with Ribena™ and the linear range of glucose observed in Figure 4.11a, indicated that the addition of 0 - 300 μ l aliquots Ribena™ corresponded to 0 - 2.22 mM glucose.

Consequently addition of 100 μ l Ribena™ (0.0618 μ A) represents 0.764 mM glucose; addition of 200 μ l Ribena™ (0.122 μ A) represents 1.462 mM glucose and addition of

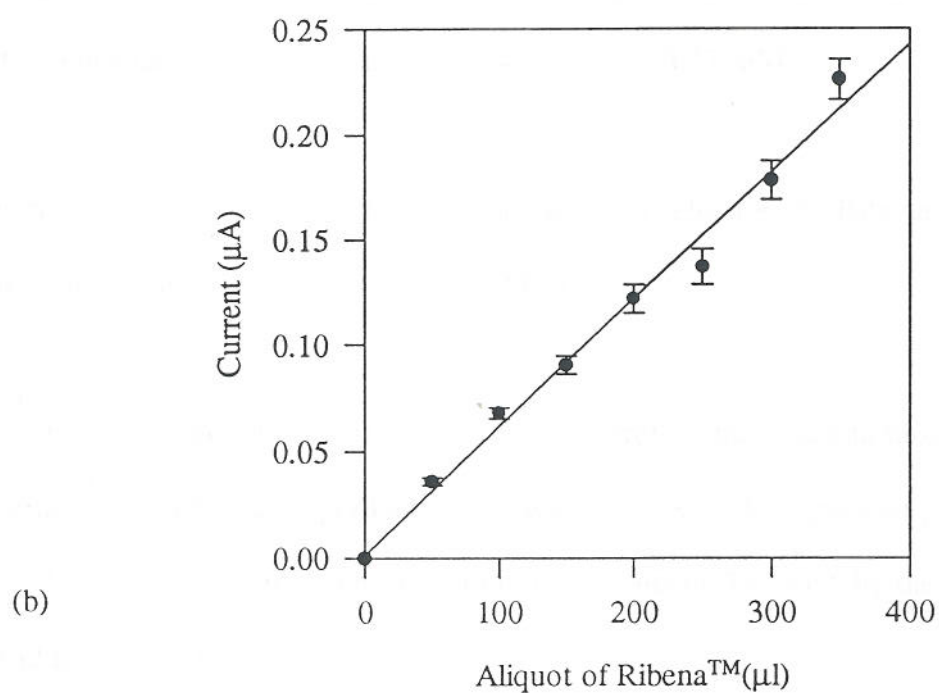
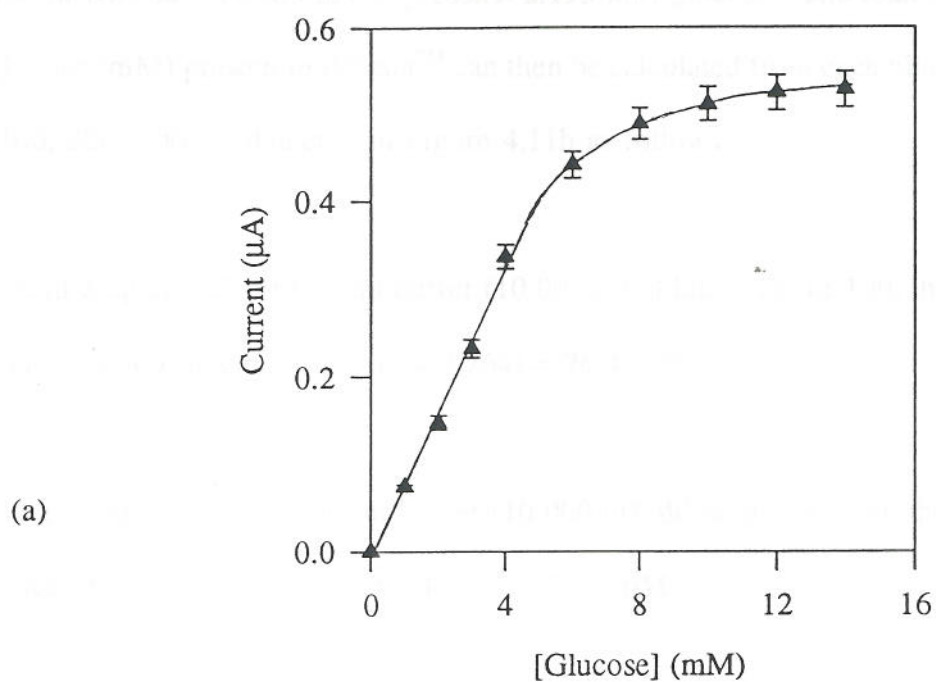


Figure 4.11. Amperometric assay of (a) glucose and (b) Ribena™ at PQQ glucose oxidase modified enzyme electrodes. Applied potential +300 mV vs SCE reference electrode. In each graph error bars represent standard deviation ($n = 3$).

300 μ l RibenaTM (0.183 μ A) represents 2.195 mM glucose. The total concentration of glucose (mM) present in RibenaTM can then be calculated from each aliquot of RibenaTM (100, 200, 300 μ l) detected in Figure 4.11b as follows:

100 μ l aliquot added to 10 ml buffer (10 000 μ l), dilution factor 100, therefore the total concentration of glucose: $(100 \times 0.764) = 76.4$ mM.

200 μ l aliquot added to 10 ml buffer (10 000 μ l), dilution factor 50, therefore the total concentration of glucose: $(50 \times 1.462) = 73.1$ mM.

300 μ l aliquot added to 10 ml buffer (10 000 μ l), dilution factor 33.33, therefore the total concentration of glucose: $(33.33 \times 2.195) = 73.17$ mM.

From these results, the mean concentration of glucose in RibenaTM, determined amperometrically, was 74.22 ± 1.89 mM, $n = 3$.

The molecular weight of glucose is 180.16, therefore the concentration of glucose in RibenaTM, determined amperometrically was 1.337 ± 0.034 g glucose per 100 ml (cf. 15g total sugar per 100 ml listed in the contents of RibenaTM by the manufacturers Smithkline Beecham).

4.3.8 UV-determination of glucose in fruit juice

The calibration curve for UV-detection of glucose using a commercial test kit supplied by Boehringer Mannheim (Figure 4.12) indicated that:

0.1 ml of 1:200 dilution Ribena™ in 3.02 ml cuvette (Δ_{A340} 0.0861) $2.37 \mu\text{g glucose.ml}^{-1}$ which corresponds to $(2.37 \times 30.2 \times 200)\mu\text{g.ml}^{-1} \equiv 0.0143 \text{ g glucose per ml Ribena}^{\text{TM}}$

0.2 ml of 1:200 dilution Ribena™ in 3.02 ml cuvette (Δ_{A340} 0.169) $4.68 \mu\text{g glucose.ml}^{-1}$ which corresponds to $(4.68 \times 15.1 \times 200)\mu\text{g.ml}^{-1} \equiv 0.0141 \text{ g glucose per ml Ribena}^{\text{TM}}$

From these results, the mean concentration of glucose in Ribena™, determined spectrophotometrically, was $1.422 \pm 0.013 \text{ g glucose per 100 ml}$.

This glucose concentration in Ribena™ determined spectrophotometrically is in good agreement with the value determined amperometrically ($1.337 \pm 0.034 \text{ g glucose per 100 ml}$). Both values also compare favourably with the glucose concentration in Ribena™ determined independently using a test kit supplied by Sigma Diagnostics: 1.383 ± 0.35 , $n = 3$ (M. F. Cardosi, personal communication).

Consequently, the PQQ-Glucose oxidase-modified enzyme electrodes prepared in this chapter can be reliably used for determination of glucose in Ribena™ black currant juice.

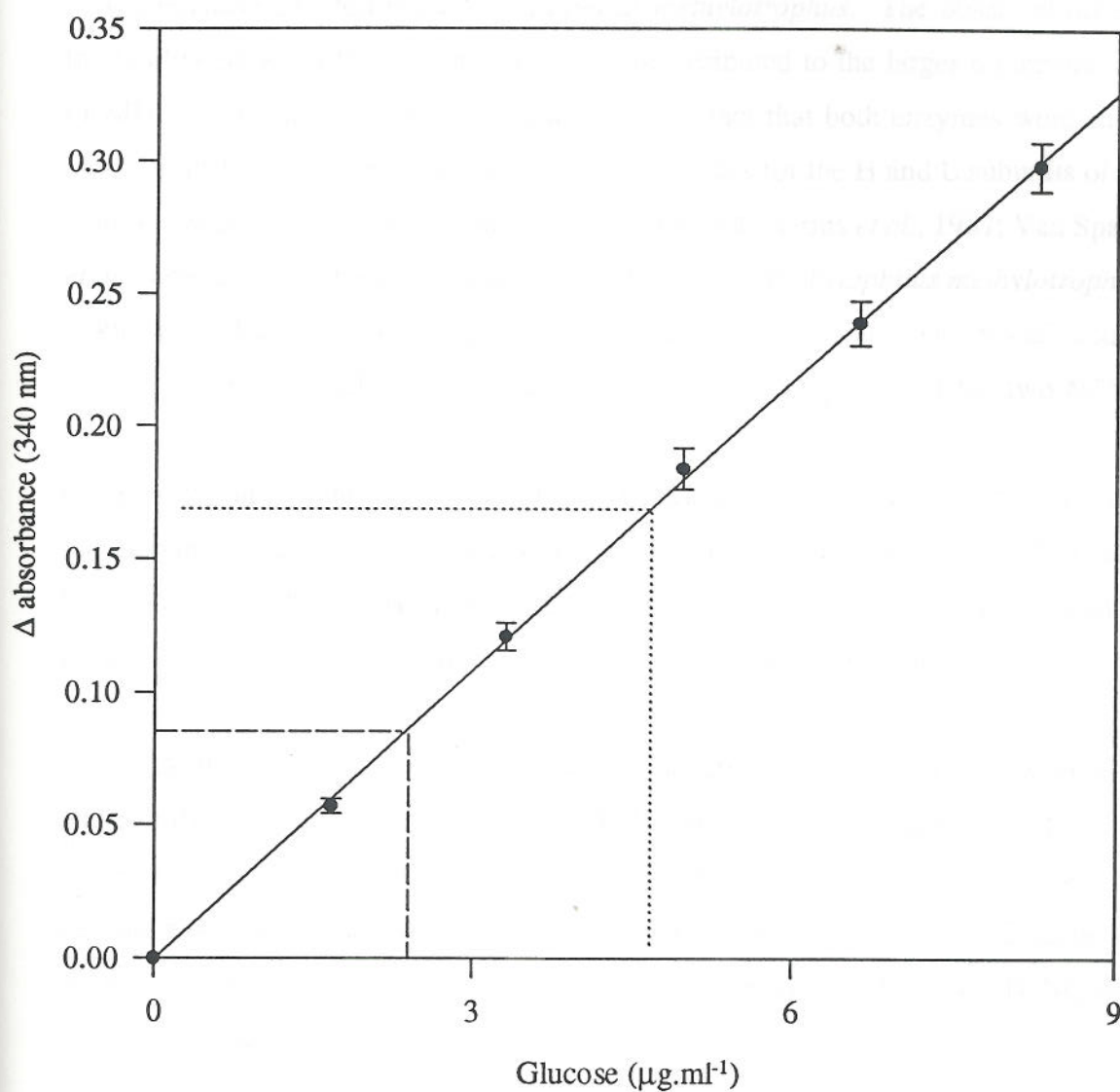


Figure 4.12 Calibration curve for UV-determination of glucose in fruit juice.

(---) represents 0.1 ml and (....) represents 0.2 ml of 1:200 dilution Ribena™

Error bars represent standard deviation (n = 3).

5.

GENERAL DISCUSSION

5.1 Stability of methanol dehydrogenase

The enzyme isolated from *Paracoccus denitrificans* is much more stable than methanol dehydrogenase isolated from *Methylophilus methylotrophus*. The observed difference in stability between the two enzymes may be attributed to the larger molecular weight of MDH from *Paracoccus denitrificans* and the fact that both enzymes were supplied in different forms. Whilst the amino acid sequences for the H and L subunits of MDH from *Paracoccus denitrificans* have been determined (Harms *et al.*, 1987; Van Spanning *et al.*, 1991), the amino acid sequences of MDH from *Methylophilus methylotrophus* are unknown. Future DNA sequencing of the latter enzyme may reveal sequence differences which could help to explain the different properties of the two MDHs.

Consecutive amperometric assays of MDH packed cavity electrodes also confirm that the enzyme isolated from *Paracoccus denitrificans* is more stable than MDH isolated from *Methylophilus methylotrophus* and can be successfully used for repeated assay of packed cavity electrodes without significant loss in current output.

Complete thermal denaturation of MDH was observed after 90 min incubation at 50°C and significant thermal denaturation of MDH within the packed cavity electrode (57% decrease in current output) occurred after 150 min incubation at 45°C. However the current response remained unchanged when assayed at 30°C and 35°C respectively suggesting that these temperatures are optimal for assay of MDH-TTF.TCNQ packed cavity electrodes.

In general, the electrode sensitivity (i.e., slope in the linear range) of MDH-TTF.TCNQ packed cavity electrodes, determined at 30°C, 35°C, 40°C and 45°C, remains relatively constant with respect to time. The optimal sensitivity at 40°C reflects the increased linear range (between 0 and 40 μM methanol) and higher current output (up to 1.7 μA) observed at this temperature. The limited linear range observed at lower temperatures ($\leq 20 \mu\text{M}$) accounts for the decreased sensitivity observed at 30°C and 35°C.

5.2 Effect of ammonium ions on MDH-TTF.TCNQ packed cavity electrodes

Determination of apparent kinetic parameters with respect to ammonium ion concentration (apparent K_A values determined to be 0.05 mM for *Paracoccus denitrificans* and 1 mM for *Methylophilus methylotrophus*) together with the increased catalytic currents observed on addition of ammonium ions to the MDH-TTF.TCNQ packed cavity electrode, indicate that ammonium ions are necessary for efficient re-oxidation of the substrate reduced MDH at the organic conducting salt electrode.

This ammonium ion activation was supported by the fact that successive current responses to methanol could only be detected at the MDH-TTF.TCNQ packed cavity electrode in the presence of ammonium ions. Whilst small currents could be detected in response to a single aliquot of methanol in the absence of ammonium ions, subsequent addition of methanol produced no further increase in current output. However, in the presence of ammonium ions successive aliquots of methanol could be assayed at the MDH packed cavity electrode. These observations are in good agreement with the absolute requirement of methanol dehydrogenase for ammonia when assayed *in vitro* with artificial electron acceptors (Duine et al., 1979; Anthony, 1982; 1990), however they contrast with the anaerobic and aerobic turnover of MDH using TTF.TCNQ in the complete absence of ammonium ions reported by Zhao and Lennox (1991).

The apparent K_M for the MDH-TTF.TCNQ packed cavity electrode was determined to be 0.02 mM (*Paracoccus denitrificans*) and 0.025 mM (*Methylophilus methylotrophus*). Both values are significantly less than the apparent K_M of 1 mM reported by Zhao and Lennox (1991) for a MDH (*Methylophilus methylotrophus*) packed cavity electrode in the absence of ammonium ions. The 50-fold reduction of apparent K_M suggests an increased affinity of the enzyme electrode for methanol substrate in the presence of ammonium ion activator. This indicates that the binding of ammonium ion activator stimulates catalysis of methanol substrate.

5.3 Detection of histamine at a TCNQ-methylamine dehydrogenase enzyme electrode

Methylamine dehydrogenase may be used for the quantitative assay of histamine and can be incorporated in a mediated enzyme electrode. The observed linear range, between 0 and 200 μM , of this amperometric histamine sensor (Figure 3.4) correlates well with elevated histamine levels prevalent in patients with chronic myelogenous leukaemia ($< 243 \mu\text{M}$, Beaven *et al.*, 1982), and the limit of detection (4.8 μM) compares favourably with the limits of detection reported for a potentiometric histamine sensitive membrane electrode (10 μM , Katsu *et al.*, 1986). Further work is required to develop a practical device and demonstrate its clinical utility.

5.4 Electrochemistry of PQQ

The absence of distinct peak shaped voltammograms during cyclic voltammetry of PQQ in solution at both platinum and gold electrodes indicates that electron transfer between PQQ and different metal electrodes is difficult. Subsequent cyclic voltammetry of PQQ at a polypyrrole modified platinum electrode (PPy/Pt) in 0.1 M phosphate buffer, pH 7.0, gave an oxidation peak at -0.116 V (vs SCE) and corresponding reduction peak at -0.225 V (vs SCE) which were attributed to the electrochemical oxidation and reduction of PQQ. However, the observed peak separation (109 mV) was much greater than the theoretical peak separation quoted for reversible electron transfer, whilst the peak potential and peak separation both increased with increasing scan rate. These observations with respect to the anodic peak current, peak separation and the effect of increasing scan rate all indicate that the electron transfer between PQQ and the polypyrrole membrane electrode is "quasi" reversible.

The quasi reversible electron transfer between PQQ and the surface of the PPy modified electrode was attributed to inefficient polymerisation of the polypyrrole and poor film growth as indicated by visual inspection of the PPy modified electrode. Possible alternatives for future investigation may involve polymerisation of pyrrole from organic solvent (e.g. acetonitrile) as opposed to aqueous 0.1 M KCl. In this respect Dicks *et al.* (1989) found that the structure of PPy films formed in aqueous electrolyte were

more coarse in appearance with a less homogeneously packed structure than PPy films formed in acetonitrile. However, Diaz (1981) previously reported that PPy films grown in anhydrous acetonitrile have a rough surface with dendrite-like structures whereas addition of as little as 1 % water to the solvent enabled growth of much smoother PPy films at the electrode surface (Diaz, 1981).

5.5 Coupling of glucose oxidase with PQQ

A significant increase in anodic current and corresponding decrease in the cathodic current was evident following the addition of glucose oxidase to a solution of PQQ and glucose in 0.1 M citrate buffer at pH 3.5. The transfer of electrons from the reduced FAD moiety of GOD to PQQ accounts for the observed increase in anodic current. Meanwhile the decrease in cathodic current arises from the simultaneous reduction of PQQ during interaction with GOD.

Investigation of coupling of PQQ with glucose oxidase at different pH values emphasize that a pH less than 6.0, is necessary for interaction between PQQ and glucose oxidase to occur and that interaction is optimal at pH 3.5. These observations are consistent with a recent report by Boeriu *et al.* who independently established, via spectrophotometry, that no reaction between PQQ and glucose oxidase could be detected in the pH range 6.0 - 8.0 whereas a three-fold increase in reaction rate was observed when the pH decreased from 5.0 to 3.0. (Boeriu *et al.* 1993).

Boeriu *et al.* suggested that the carboxylic acid groups of PQQ with a pKa of around 5.0 must be protonated before the appropriate structural interaction between the quinone moiety of PQQ and the FAD moiety of GOD can occur. The resultant charge transfer complex formation facilitates electron transfer from the reduced FAD moiety of glucose oxidase to PQQ prior to glucose catalysis (Boeriu *et al.*, 1993).

The inability of PQQ-PPy modified electrodes to couple with glucose oxidase, the inefficient polymerisation of polypyrrole and the "quasi" reversible electron transfer of PQQ observed at PPy modified electrodes indicate that PPy membrane electrodes are

not suitable for future development of PQQ modified enzyme electrodes.

However the coupling of PQQ and glucose oxidase at pH 3.5 enabled the development of PQQ-GOD modified enzyme electrodes for detection of glucose in RibenaTM black currant juice. The corresponding amperometric determination of glucose (1.337 ± 0.034 g per 100 ml RibenaTM) was in good agreement with concentration determined using commercial enzymatic test kits for glucose supplied by Boehringer Mannheim and Sigma Diagnostics.

5.6 Further work

This research has confirmed that the quinoprotein methylamine dehydrogenase may be incorporated into an enzyme electrode for detection of histamine. In order to use this TCNQ modified electrode for the detection of histamine levels prevalent in chronic myelogenous leukaemia the effect of polyamines such as spermine, spermidine and putrescine should be evaluated. Further work is also necessary to ascorbic acid interference at TCNQ-MADH electrodes (60 % of the observed response to histamine Table 3.2). Of the techniques available to reduce or eliminate ascorbic acid interference at different enzyme electrodes, use of selectively permeable membranes such as Nafion[®] and cellulose acetate (Dong et al., 1991; Lowry & O'Neill, 1992) appears the most practical solution since this would provide an external barrier to exclude ascorbic acid from the electrode surface. Use of platinised carbon and/or chemically modified electrode surfaces could enable enzyme electrodes to be operated at a lower applied potential where ascorbic acid interference is minimised (White et al., 1994, Jaffari 1994). However, since the electrode surface is already modified with TCNQ and then coated with MADH enzyme, further chemical modification of electrode surface may adversely affect the efficiency of TCNQ to mediate electron transfer, whilst platinization of the carbon electrode surface may be complicated and time consuming.

PQQ has been successfully employed to mediate electron transfer within a PQQ-glucose oxidase electrode for detection of glucose in fruit juice. However since the transfer of electrons between glucose oxidase and PQQ is dependent on the protonation of PQQ

at $\text{pH} \leq 5$, this system is not suitable for detection of glucose at $\text{pH} 7$ and cannot be applied for physiological glucose analysis. However, cystamine modified PQQ electrodes have already been developed for the NAD(P)^+ dependent enzyme malic dehydrogenase (Willner & Riklin, 1994). Future use of the NAD(P)^+ dependent glucose dehydrogenase within a PQQ modified enzyme electrode may provide an alternative sensor for analysis of glucose at physiological pH . This may also overcome the problems associated with electrode fouling at high NADH concentrations (Tse & Kuwana, 1978).

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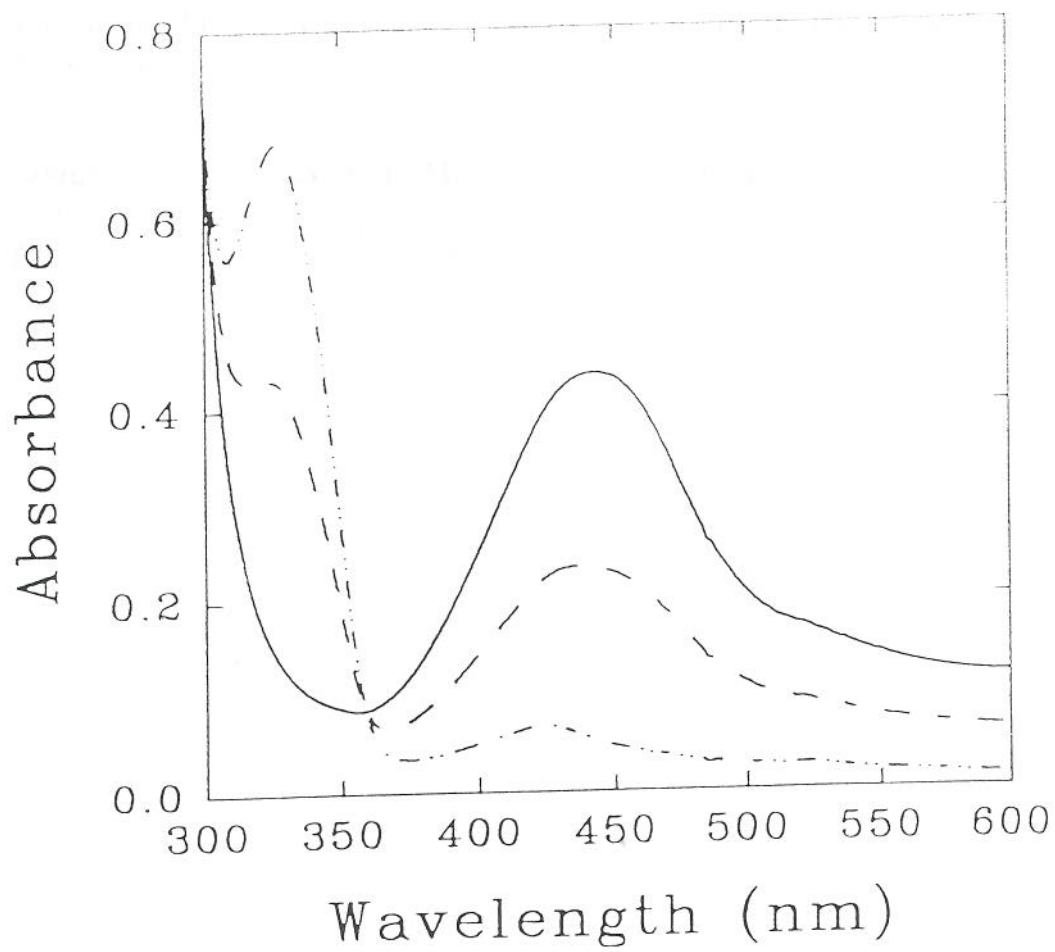
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APPENDIX ONE

Spectral change observed after addition of histamine dihydrochloride to methylamine dehydrogenase (V.L. Davidson, University of Mississippi Medical Center, personal communication). Methylamine dehydrogenase ($16.7\ \mu\text{M}$) was present in 50 mM potassium phosphate, pH 7.5 at 30°C .

Spectra were recorded of oxidized methylamine dehydrogenase (—), 2 min after addition of 1 molar equivalent of histamine (----) and 8 min after addition of a second molar equivalent (-...-).



PUBLICATIONS

Work from this thesis has been reported in the following papers:

Loughran, M. G., Hall, J. M., Turner, A. P. F. & Davidson, V. L. (1995). Amperometric detection of histamine at a quinoprotein dehydrogenase enzyme electrode. *Biosensors & Bioelectronics* 10: 569-576.

Loughran, M. G., Hall, J. M., Turner, A. P. F. & Davidson, V. L. (1995). Ammonium ion requirement and stability of methanol dehydrogenase TTF.TCNQ electrodes. *Analytical Letters*. Submitted.

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